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Ecotoxicological threshold levels of a mixture of herbicides (atrazine, diuron and metolachlor) in freshwater microcosms

Elizabeth M. Hartgers[1,3,**], G.H. (René) Aalderink[2,3], Paul J. Van den Brink[2], Ronald Gylstra[1], J. Wilfred F. Wiegman[1,2] and Theo C.M. Brock[2*]

[1]*Wageningen Agricultural University, Department of Water Quality Management and Aquatic Ecology (LUW-WKAO), P.O. Box 8080, 6700 DD Wageningen, The Netherlands (Fax.: +31 317 484411);* [2]*DLO Winand Staring Centre for integrated Land, Soil and Water Research (SC-DLO), P.O. Box 125, 6700 AC Wageningen, The Netherlands (Fax.: +31 317 424812);* [3]*National Institute of Public Health and the Environment (RIVM), P.O. Box 1, 3720 BA Bilthoven, The Netherlands. (Fax.: +31 30 2742971);* ***Present address: DLO Netherlands Institute of Fisheries Research (RIVO-DLO), P.O. Box 68, 1970 AB IJmuiden, The Netherlands*

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Abstract

Twelve indoor, plankton-dominated, freshwater microcosms (600 l) were used to study the effect of a mixture of herbicides on structural and functional aspects of these ecosystems. The $EC_{50,\ 72\ h}$ values of the most susceptible standard test alga *Selenastrum capricornutum* ($EC_{50,\ atrazine} = 54\ \mu g\ l^{-1}$, $EC_{50,\ diuron} = 15\ \mu g\ l^{-1}$, $EC_{50,\ metolachlor} = 56\ \mu g\ l^{-1}$) were used as a starting point for the dosage applied in the microcosms (dosages: 0, 0.01, 0.03, 0.1, 0.3, 1× EC_{50}). The microcosms were exposed to chronic levels for 28 days and subsequently monitored for 4 more weeks.

The following effects were observed: (1) direct effects became apparent from an initial drop in photosynthesis efficiency, pH and oxygen concentration and a decrease in the abundance of several phytoplankton taxa at the 0.3 × EC_{50} treatment level and higher. (2) Fourteen days post application an increase in the abundance of several phytoplankton taxa (*Chlamydomonas* sp. and *Stephanodiscus/Cyclotella)* was observed; oxygen concentrations recovered while alkalinity, conductivity and total inorganic nitrogen were elevated. (3) Effects on fauna were minor. *Daphnia galeata* showed a decreasing trend and the cyclopoid copepods an increasing trend at the end of the experiment.

Multivariate statistical analyses demonstrated no effects of any treatment level on the zooplankton community. Effects were reported for the phytoplankton community at dose levels of 0.3 × EC_{50} and higher. On species level the most sensitive taxon was Chlorophyceae coccales. For this taxon a NOEC at the dose level of 0.01 × EC_{50} was calculated. This effect however was relatively small in magnitude and merely based on an increase in numbers in the control and lowest treated microcosms rather than a decrease in numbers in all other treatments. The standards based on algal toxicity data, as adopted by the Uniform Principles, consist of a safety factors of 0.1 to be multiplied with the EC_{50}. The NOEC of coccales was lower than 0.1 × EC_{50}. All other observed variables in this aquatic ecosystem were sufficiently protected against the mixture of herbicides by the safety factor as proposed in the Uniform Principles.

Introduction

Pesticides used for crop protection, or for green space management in urban areas, may enter the aquatic ecosystem via spray drift, leaching, surface run-off or accidental spills. Pesticides are regularly detected in surface waters in the Netherlands, even at concentrations above levels permissible in the Netherlands (Phernambucq et al., 1996) and increasingly, the prob-

lem of combination toxicity in freshwater systems arises. Active ingredients may be sold together within one formulation, e.g., atrazine and metolachlor. In addition, several herbicides are relatively persistent compounds and therefore can occur widely downstream of catchment areas where they may combine with other pesticides. It is largely unknown whether the presence of combinations of pesticides in freshwater ecosystems results in greater adverse effects than would be expected on the basis of their separate toxicities.

In 1997, the member states of the European Union adopted the uniform principles concerning the marketing of crop protection products (EU, 1997). In general, this directive states that the Predicted Environmental Concentration (PEC) of individual pesticides in surface water should not exceed 0.01 times the acute EC_{50} (concentration at which 50% of the test organisms show an effect) or 0.1 times the chronic NOEC (No Observed Effect Concentration) of the standard test species *Daphnia* or fish, or 0.1 times the EC_{50} (based in inhibition of growth) of the standard test algae. The Dutch legislation only differs with respect to the algae; the NOEC of algae instead of the EC_{50} is used as toxicological endpoint. The current standards are based on experiments with single substances and on a limited number of aquatic test species, representative of several trophic levels in the aquatic ecosystem, which are easily kept in the laboratory. The lack of cost-effective alternatives (Van Leeuwen et al., 1994) is used to justify the use of these standardized single species toxicity tests. As the safety factor is crucial in ensuring an adequate protection of the freshwater environment (La Point & Perry, 1989; Persoone & Janssen, 1994), model ecosystem experiments should be performed to validate these safety factors. Although smaller and less complex than real-world ecosystems, freshwater microcosms provide a tool for performing ecosystem research in test systems under standardized conditions, which are manageable in terms of costs and logistics and can be replicated (Giddings, 1980). Such experiments have been performed for a large group of individual substances (e.g., Brock & Budde, 1984). However, very little information is available on the effects of mixtures of pesticides on a freshwater community.

The present paper describes the effects of a chronic application of a mixture of three herbicides (atrazine, diuron and metolachlor) on model ecosystems. The chronic application (28 days) used in the experiment represents a worst-case scenario. The herbicides selected are representatives of three chemical groups and two modes of action. All substances inhibit the primary producers. Atrazine and diuron inhibit photosynthesis via blockage of the electron transport in the photosystem II (PSII) (Van Rensen, 1989). Metolachlor affects several physiological processes in the algal/plant cell such as the lipid-protein synthesis thus indirectly inhibiting respiration and photosynthesis (Chesters et al., 1989). In 1993, atrazine, diuron and metolachlor were regularly measured in the large rivers and lakes of the Netherlands, with reported maximum levels of 0.7, 0.6 and 0.02 μg l^{-1}, respectively (Phernambucq et al., 1996). The worst case environmental (acute) concentrations in agricultural ditches near sites of application are probably considerably higher ($> 0.1 \times EC_{50}$).

The aims of the present paper are: (1) to describe effects caused by the application of a mixture of herbicides on the functioning and structure of the ecosystem in the microcosms; (2) to compare these results with the effects observed in more or less similar experiments performed previously with individual herbicides; and (3) to validate the safety factors of the Uniform Principles in the case of a chronic exposure to a mixture of herbicides.

Materials and methods

Microcosms

Twelve macrophyte-free microcosms, hereafter referred to as 'microcosms', were installed in two rows in a climatized room (water temperature approximately 20 °C). Each cosm consists of a glass aquarium (length $\times$ width $\times$ height $= 1.1 \times 1.1 \times 0.7$ m^3; water volume $= 600$ l) which was filled four weeks before the start of the experiment (acclimatization period) with 10 cm of sandy loam lake sediment, and 50 cm of well water. The evaporated water was replenished at weekly intervals. Artificial daylight (photoperiod 14 h) was provided by Philips HPI-T 400 W high-pressure metal lamps, resulting in an average irradiance at the water surface of 120 μE m^{-2} s^{-1}. During the acclimatization period the microcosms were interconnected by tubes ($\varnothing = 2.6$ cm) and the water was circulated by means of a pump at a rate of 3.8 l min^{-1} to ensure similar ecosystem conditions in all microcosms. The introduction of some salt into one of the microcosms after the experiment and the installation of two conductivity meters in the first and the last cosm

revealed that the salt concentrations, and therefore the degree of water mixing in all systems, were equal after 55 h.

On April 29, 1996 (day −14) the connecting tubes were removed and the actual experiment started. Throughout the experiment, an aquarium pump was used in each cosm to circulate the water slowly (3.8 l min^{-1}). The salt application after the experiment revealed that the water column within each cosm was mixed in approximately 1½ h. Nutrients were added to all microcosms as KH_2PO_4 and NH_4NO_3 to a level of 90 μg N l^{-1} and 15 μg P l^{-1} (N:P = 6 : 1). Before the start of the experiment, nitrogen and phosphorus had been supplemented twice a week, while during the experiment, mainly nitrogen was added weekly, up to water concentrations of 150 μg l^{-1}, to avoid a bloom of nitrogen-fixing cyanobacteria. At several moments during the acclimatization period, plankton and macro-invertebrates typical of Dutch water systems were introduced. The macro-invertebrates comprised several taxonomic groups representing various trophic levels. For a more detailed description of the sediment, microcosms and equipment, see Brock et al. (1992a).

Treatment

The $EC_{50,\ 72\ h}$ values for *Selenastrum capricornutum*, as assessed by Ietswaart (pers. comm.) according to OECD guidelines, were used as a starting point for setting dosages in the microcosms ($EC_{50,\ atrazine} = 54$ μg l^{-1}, $EC_{50,\ diuron} = 15$ μg l^{-1}, $EC_{50,\ metolachlor} = 56$ μg l^{-1}). The following dosages of each of the three herbicides were applied, in two microcosms each: 0, 0.01, 0.03, 0.1, 0.3 and 1 × EC_{50}. The treatments were divided randomly among the microcosms. This established a concentration range of a cocktail of three herbicides, in which the proportion of the herbicides was always similar, a regression design which allowed the assessment of threshold levels for this mixture of herbicides.

The experimental design included three periods: (1) the pre-treatment period (day −7), used to describe the initial conditions of the systems; (2) the treatment period (days 1 to 28), during which the concentrations of the herbicides in the water phase were kept at a constant level, and (3) the post-treatment period (days 29–49), used to observe the degradation of the herbicides in the water phase and the long-term effects of the treatment on the different elements of the ecosystem in the microcosms. During the experimental period, all endpoints were measured at weekly intervals except the periphyton chlorophyll-*a* concentration (weekly from day 3 onwards) and the macro-invertebrate population (day −5 and day 52).

Application of herbicides

The herbicides atrazine, diuron and metolachlor were applied as Pestanal® analytical standards (Riedel-de Haën AG). The purities of atrazine (6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine, lotnr.: 20350), diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea, lotnr.: 51080) and metolachlor (2-chloro-6′-ethyl-N-(2-methoxy-1-methylethyl)acet-o-toluidide, lotnr.: 41790) were 98%, 99% and 97% respectively. For physical and chemical information on the herbicides, see Tomlin (1994). Stock solutions of the herbicides were prepared by dissolving them in 1 ml acetone. Introduction of the herbicides into the cosm was carried out by mixing the required volume of the stock solution with approximately 10 litres of tap water. After this solution had been added to the cosm, the water column was stirred with a glass rod. The control microcosms were also treated with 1 ml acetone. During the chronic exposure period of 28 days, water samples representative of the entire water volume were analyzed regularly at 10 sampling dates. After analysis, the herbicides were supplemented if necessary in order to maintain nominal herbicide concentrations.

Analysis of the herbicides

The herbicides in the water samples were concentrated by a solid phase extraction technique before HPLC analysis. Solid phase extraction columns were prepared by filling a cartridge (5 ml) with 0.4 g octadecyl material (Bakerbond C-18 40 μm, lotnr.: 7025-01, J.T. Baker inc.), fixed between two propylene filters. These columns were conditioned with 5 ml methanol followed by 5 ml distilled water. After extraction of a known volume of water, the herbicides were eluted from the column with three times 0.5 ml of acetonitrile. Finally, the samples were diluted with distilled water to a volume of 5 ml and subsamples were transferred to HPLC-vials for analysis. Recovery rates of atrazine, diuron and metolachlor from spiked water samples were 109% (s.d. = 3.6%; $n = 10$), 99% (s.d. = 2.1%; $n = 5$) and 107% (s.d. = 2.2%; $n = 10$), respectively.

Atrazine and diuron were analyzed using a Waters Novapak C-18 (length: 150 mm; internal diameter:

4.6 mm; particle size: 4 μm) analytical column, provided with a guard column of the same origin. The mobile phase consisted of a mixture of water and acetonitrile at a ratio of 50:50 (v/v), adjusted to a flow rate of 1.0 ml min^{-1}. Metolachlor was analyzed separately using a Merck Lichrocart C-8 (length: 125 mm; internal diameter: 4 mm; particle size: 5 μm) analytical column, provided with a guard column of the same origin. The mobile phase consisted of a mixture of water and acetonitrile at a ratio of 70:30 (v/v), adjusted to a flow rate of 1.3 ml min^{-1}. Both columns were mounted in a Waters TCM column oven, which was set at a temperature of 40 °C. The HPLC-pump used was a Waters model 510. Samples of 150 μl were injected with an Perkin Elmer ISS100 autosampler, while the herbicides were detected using a Perkin Elmer LC-90 UV-detector set at a wavelength of 214 nm. The detection limit of all herbicides was 0.01 μg l^{-1}. The concentrations of the herbicides were calculated using an Area-Under-the-Curve method (Van Wijngaarden et al., 1996).

Physical/chemical endpoints

At weekly intervals, after light over the microcosms had been switched on for 6 to 8 hours, dissolved oxygen (WTW Oxi 196 oxygen meter and WTW EOT 196 oxygen probe), pH (Metrohm (E-588), conductivity (WTW LF 191) and alkalinity were measured at a water depth of approximately 10 cm. Alkalinity was estimated by titration of a 100 ml sample with 0.05 M HCl down to pH 4.2. These variables are indicators of community metabolism and of ecosystem health (Kersting, 1994). In order to obtain a representative sample of the water column, five depth-integrated samples were taken at different positions within the cosm using a perspex tube (length 40 cm, volume 1 l). Of this 5 l sample, 100 ml was used for an analysis of the nutrient concentrations (nitrate, ammonium and orthophosphate), 1 l was used to study the phytoplankton species composition, 1 l to assess the chlorophyll-*a* content and 100 ml to determine the bacterial concentration in the water phase. The 100 ml sample was stored at −20 °C after filtering over a 0.45 μm membrane filter. The nutrient concentrations were determined using a DIONEX AI-450 ion-chromatograph. The compounds were separated with a DIONEX AS4A-column and detected with a Pulsed Electrochemical Detector. In the data analysis, nitrate and ammonium concentrations were combined to total inorganic nitrogen.

Phytoplankton

The phytoplankton sample was stained with 0.5% Lugol solution and stored in bottles to allow sedimentation. After seven days, 90% of the water volume was siphoned off and the remaining sample was preserved with 2% formalin. Cell counts were made using an inverted microscope and 40 × 40 × 4 mm sedimentation chambers. The number of individuals per species in each sample was estimated by counting a fixed volume containing at least 100–300 individuals (Lund et al., 1958).

For the chlorophyll-*a* analysis, one litre of water was filtered over a Whatman GF/C glass fibre filter. The chlorophyll-*a* content of the filter was analyzed spectrophotometrically (665 nm) after ethanol extraction at 70 °C (Moed & Hallegraeff, 1978). Between sampling and analysis, the filters were stored for less than two months at −20 °C.

The efficiency of photo-system II electron transport was determined from fluorescence measurements with a slightly modified version of the Xe-PAM fluorometer. A detailed description of equipment and measuring techniques can be found in Snel et al. (1998). Before measuring the efficiency of the PSII electron transport of a representative sample of the algal community in the microcosms, the algae were acclimatized for 20 min at 20 °C and an irradiation level of 120 μE m^{-2} s^{-1}.

Periphyton

Periphyton was sampled from glass slides serving as artificial substrates. The glass slides were positioned vertically in a perspex frame just below the water surface. The incubation time of the slides was four weeks. On each sampling date of the periphyton, 3 slides were removed from the frame for chlorophyll-*a* analysis. The slides were brushed until visually clean and the periphyton removed was collected in tap water. This algal suspension was concentrated over a Whatman GF/C glass fibre filter and chlorophyll-*a* was analyzed as described above.

Bacterioplankton

100 ml of the sample taken for nutrient and phytoplankton analysis was used for the bacterial analysis.

Bacterial abundance was determined by epifluorescence microscopy. The bacteria were stained with 10 μM of the fluorochrome Hoechst 33258 (Sigma) and filtered onto blackened polycarbonate membranes (Poretics) with a pore size of 0.2 μm. The filter was embedded in immersion oil on a glass slide. The number of bacteria in at least 10 microscope fields was counted, using a Zeiss IM35 inverted microscope (magnification: 1000×).

The bacterial activity was measured by the [3H]thymidine incorporation method (Fuhrman & Azam, 1982), following the procedure described by Tubbing & Admiraal (1991). The specific growth rate of the bacteria was calculated assuming a conversion factor of 10^{18} cells formed per mole of thymidine incorporated (Smits & Riedmann, 1988).

Zooplankton

The species composition of the zooplankton was studied by concentrating a 5 l sample, taken in the same manner as the nutrient and phytoplankton sample, using a nylon zooplankton net (mesh size 55 μm) and preserving in 4% formalin. The large specimens were counted and identified using a stereo-microscope. A sub-sample was used to count and identify the smaller specimens using an inverted microscope and a 4 ml counting chamber until at least 300 individuals had been counted.

Macro-invertebrates

Macro-invertebrates were sampled by means of artificial substrates (pebble baskets and multiplates). Since herbicides were applied in low dosages, no direct effects on macro-invertebrates were expected. Most of the macro-invertebrates present were long-living species able to adapt to poor food conditions, so no severe indirect effects were expected either. Therefore, only two samples were taken (day -5 and 52). The pre-treatment sample was taken to verify that the fauna was distributed evenly among the microcosms while the post treatment sample was used to observe long-term effects on the macro-invertebrates from the application of the herbicides. For details on the sampling method, see Brock et al. (1992b).

Data analysis

Transformations

To downweight high abundance values and obtain a normal distribution of the data, the phytoplankton and zooplankton data sets were $\ln(x)$ transformed prior to analysis. The phytoplankton and zooplankton data sets were respectively $\ln(0.0005x + 1)$ and $\ln(10x + 1)$ transformed, where x stands for the abundance value (for rationale, see Van den Brink et al., 1995).

Univariate analysis

The Williams test (Williams,1971, 1972) was used to compare values of control and treated microcosms in order to assess a No Observed Effect Concentration (NOEC; $p \leq 0.05$). The test assumes that the mean response of the variate is a monotonic function of the treatment, thus expecting an increasing effect with increasing dose. In this paper, the results of the Williams test are considered valid if treatment effects can be observed for at least two consecutive sampling dates. The test was performed using the Community Analysis, version 3.5 software package (Hommen et al., 1994). The analysis was performed on the individual phytoplankton, zooplankton and macro-invertebrate taxa, and on physical and chemical variables. The analysis results in an overview of NOECs in each sampling week for the data analyzed.

Multivariate analysis

The phytoplankton and zooplankton data sets were analyzed using the Principal Response Curves method (Van den Brink & Ter Braak, acc.). Principal Response Curves (PRC) are based on redundancy analysis (RDA), the canonical form of principal component analysis (PCA). The PRC technique can be used, like other multivariate techniques, to summarize all information on the investigated populations simultaneously and thus elucidate effects of contaminants at community level. This analysis results in a diagram showing the multivariate response of the treatments in time with respect to the control. Figure 2 provides an example. In this diagram, the sampling date is expressed on the horizontal axis and the deviation of all treatments with respect to the control on the vertical axis. The species weights (b_k) as shown on the right side of the figure can be interpreted as the affinity of each species with the diagram. For instance, in Figure 2 *Chlamydomonas* sp. has a positive weight in the diagram which implies that its abundance increased at the two highest treatment levels (see Results section). *Monoraphidium* sp. has a negative weight and thus decreased at these treatment levels. For theoretical

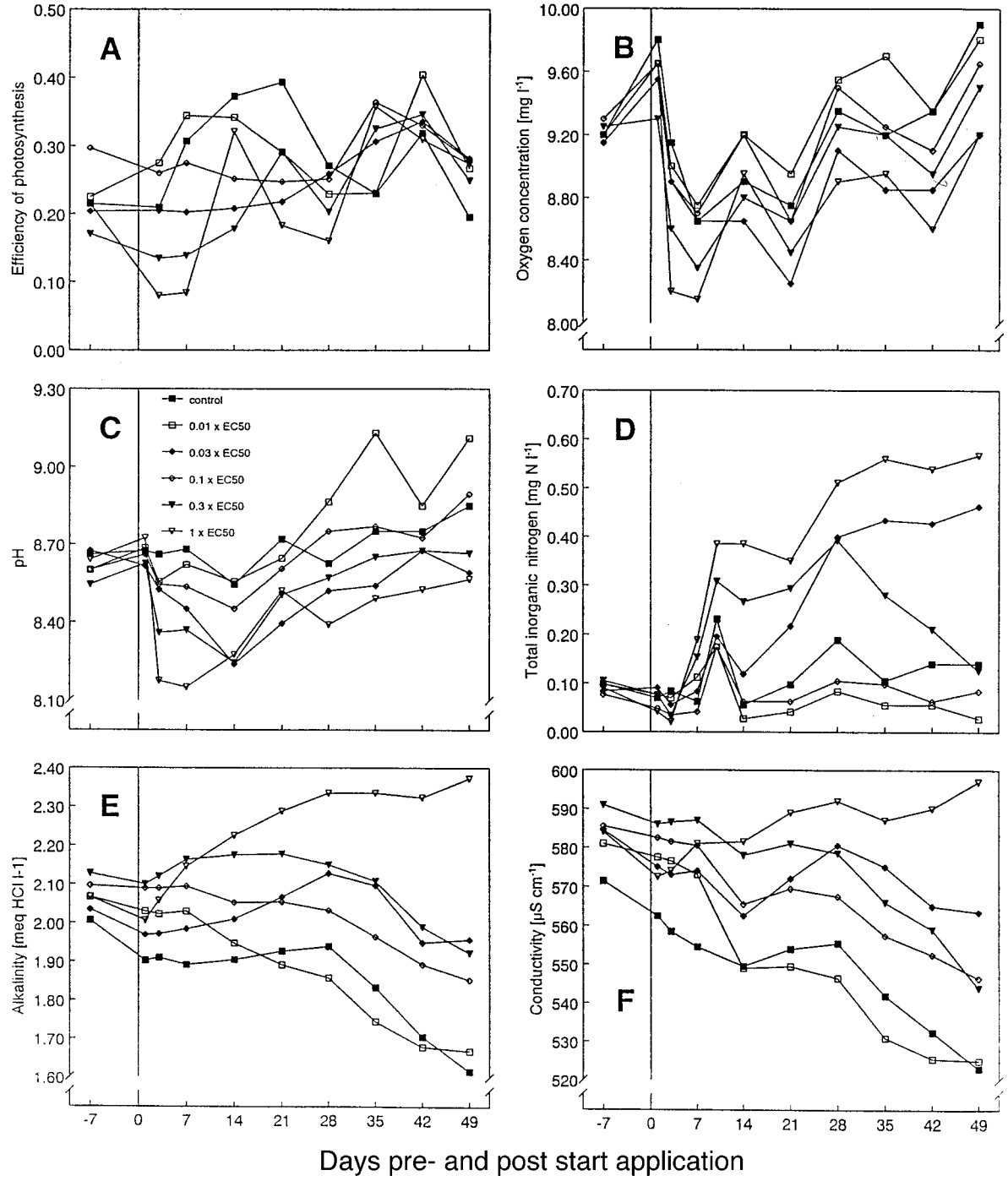

Figure 1. Dynamics of community metabolism endpoints in the microcosms based on the geometric means of the variable per treatment level: efficiency of photosynthesis (A), dissolved oxygen concentration (B), pH (C), total inorganic nitrogen (D), Alkalinity (E) and Conductivity (F).

background, technical details and a discussion of the PRC analysis see Van den Brink & Ter Braak (1997, and acc.). All multivariate analyses were performed using the CANOCO version 3.1 software package. (Ter Braak, 1988).

The significance of the treatment effects were tested using two different Monte Carlo permutation tests. The F-type criterion was used to test whether the PRC diagram as a whole contained significant information (Ter Braak, 1990; Van den Brink & Ter Braak, acc.). To test at which sampling dates the communities showed significant effects, Monte Carlo permutations were also performed as well for each individual sampling date. For a detailed discussion of the application of Monte Carlo permutation in model ecosystem experiments, see Van den Brink et al. (1996) and Van Wijngaarden et al. (1995).

Once a variable has been calculated that best summarizes the community variance, the Williams test, used in univariate analysis, can be used to calculate a $NOEC_{community}$. PCA was used to calculate the first principal component, which is the single variable that best summarizes the community variation. This variable is a linear combination of the species data, not *a priori* related to the toxicant. PCA was performed on the data sets for each sampling week. The principal component of the samples was analyzed using the Williams test. These analyses resulted in a $NOEC_{community}$ for each sampling week (Van den Brink et al., 1996).

Results

Herbicide concentrations in the water column

Table 1 summarizes the average concentrations of the herbicides in microcosms at each treatment level during the treatment period (day 0-28). The concentrations measured were generally slightly lower than those aimed at. The concentrations in the pairs of microcosms receiving the same treatment were very similar and generally constant over the exposure period. Table 2 presents the half-life values for the disappearance of the herbicides from de water phase ($t_{1/2}$), based on the post treatment period and the final concentrations at day 49, calculated on the assumption of a first order degradation process. Diuron and metolachlor had a relatively short $t_{1/2}$ ($t_{1/2}$ diuron = 6–39 days; $t_{1/2}$ metolachlor 13–37 days) whereas atrazine was relatively persistent ($t_{1/2}$ 102–225 days) (Table 2). The herbicides showed a dose-dependent disappearance, which was faster at low concentrations.

Physical/chemical endpoints

Table 3 and Figure 1 summarize the main effects of herbicide application on community metabolism and water quality endpoints. These data were analyzed using the Williams test for a significant treatment effect compared to the control microcosms. On the first sampling date after the application of the herbicides, photosynthesis efficiency of the phytoplankton dropped in the microcosms treated with the two highest dosages (Table 3, Figure 1A). This drop in photosynthesis efficiency was also reflected in a slightly decreased oxygen level (Table 3, Figure 1B) and pH (Table 3, Figure 1C) of the water. After 14 days a recovery of photosynthesis efficiency, oxygen concentration and pH could be observed, followed by a new decrease, on days 21–28, of photosynthesis efficiency and, to a lesser extent, of oxygen concentration and pH. On all sampling dates, however, the oxygen level during the daytime remained above 8 mg l^{-1}, ensuring support for all relevant biological processes. From day 7 onwards, more inorganic nitrogen (Table 3, Figure 1D) was available at the highest treatment level, whereas dissolved orthophosphate showed no significant change at any time (not shown). Alkalinity (Table 3, Figure 1E) showed a significant increase at the highest treatment level from day 14 onwards, followed by an increase in conductivity (Figure 1F) from day 35 onwards. An average water temperature of 19.7 °C (min.: 18.1 °C; max.: 23.2 °C) was maintained. The individual microcosms never varied more than 2.1 °C from the other microcosms on the same sampling date. On day 21 the temperature in all systems was 2–3 degrees higher, than in all other sampling weeks (to app. 23 °C), due to a temporary malfunction of the cooling installation.

Thus, significant effects on the community metabolism caused by application of the herbicides could, in general, be observed at concentrations of 0.3 and 1 × EC_{50} (Table 3). The $NOEC_{community\ metabolism}$ was 0.1 × EC_{50}.

Algal community

Chlorophyll-a

Although the effects of herbicides on community metabolism were evident at high dosages, this was less pronounced in the analysis of the chlorophyll-*a*

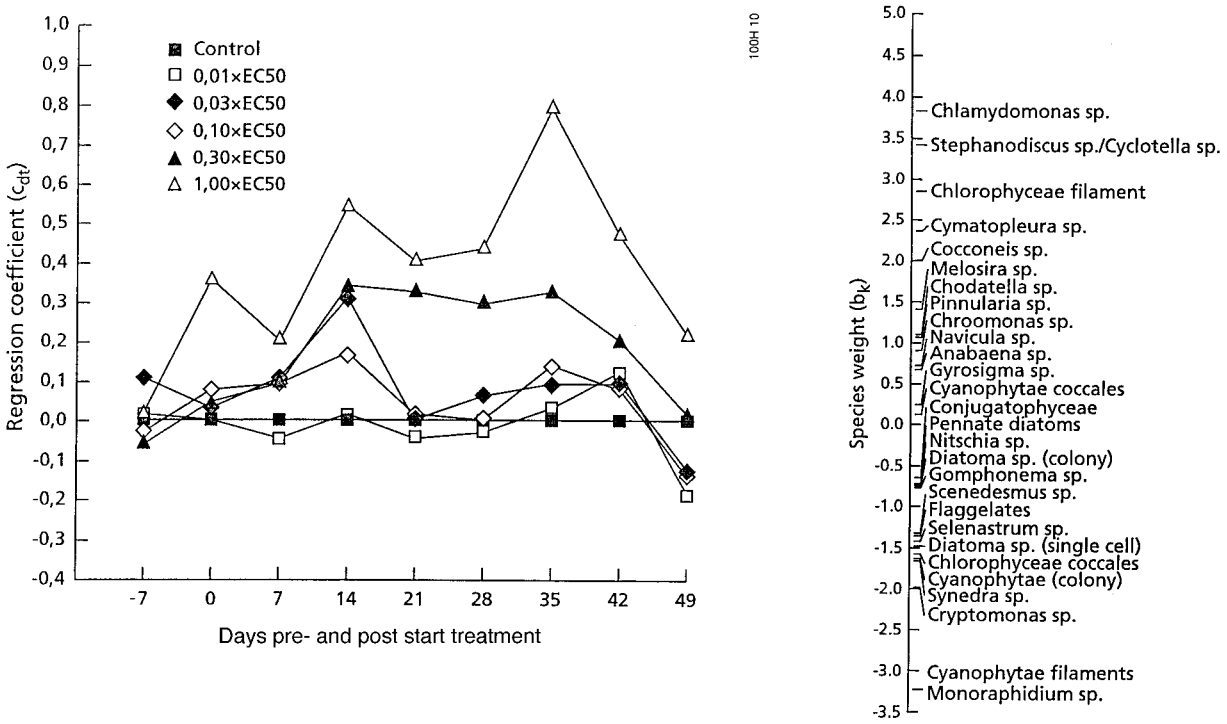

Figure 2. Principle Response Curve (PRC) of phytoplankton community. The deviance of each of the treatment levels from the control is depicted. The species weights, shown on the side of the plot, indicate the affinity of each individual taxon with the PRC. A positive species weight indicates that the abundance of the species increased in the highest two treatments.

Table 1. Average herbicide concentrations in the water column (μg l^{-1}) and their standard deviations (between brackets) during the treatment period (day 0–28) of the microcosm experiment. Average concentrations were calculated by an Area Under the Curve Method (Van Wijngaarden et al., 1996). During the treatment period replicate samples in each microcosm were taken at 10 different days

Treatment level	Atrazine		Diuron		Metolachlor	
	Target conc.	Measured conc.	Target conc.	Measured conc.	Target conc.	Measured conc.
0.01*EC50	0.54	0.55 (0.04)	0.15	0.14 (0.03)	0.56	0.56 (0.07)
		0.55 (0.04)		0.13 (0.03)		0.54 (0.06)
0.03*EC50	1.62	1.49 (0.06)	0.45	0.42 (0.06)	1.68	1.52 (0.14)
		1.58 (0.07)		0.43 (0.08)		1.52 (0.12)
0.1*EC50	5.4	4.96 (0.2)	1.5	1.39 (0.15)	5.6	4.89 (0.37)
		4.66 (1.08)		1.38 (0.35)		5.1 (0.42)
0.3*EC50	16.2	14.31 (0.41)	4.5	4.09 (0.37)	16.8	14.48 (1.41)
		14.3 (0.42)		4.47 (0.33)		15.25 (1.06)
1*EC50	54	47.38 (0.72)	15	14.11 (0.74)	56	49.81 (3.62)
		48.23 (1.21)		14.43 (0.59)		50.12 (3.7)

Table 2. Half life ($t_{1/2}$) of the disappearance of the herbicides atrazin, diuron and metolachlor in days and between brackets their final concentrations at day 49 (in μg l^{-1}) in each microcosm. $t_{1/2}$ was estimated from data of the post-treatment period (day 28–49) and calculated on the assumption of first order degradation

Treatment level	Atrazin	Diuron	Metolachlor
0.01 × EC50	102 (0.40)	14 (0.04)	16 (0.19)
	116 (0.41)	6 (0.01)	13 (0.24)
0.03 × EC50	117 (1.20)	9 (0.05)	25 (0.86)
	147 (1.36)	12 (0.09)	28 (0.80)
0.1 × EC50	152 (3.96)	21 (0.66)	18 (1.82)
	180 (4.07)	30 (0.69)	29 (3.05)
0.3 × EC50	221 (12.3)	16 (1.13)	23 (6.28)
	206 (11.8)	39 (2.51)	37 (9.52)
1 × EC50	225 (41.6)	25 (6.64)	37 (33.7)
	214 (41.6)	37 (8.18)	32 (31.5)

content of the primary producers. The chlorophyll-*a* concentration of both periphyton and phytoplankton showed a high level of variability (phytoplankton: avg. 52 μg l^{-1}; min. 6 μg l^{-1}; max. 222 μg l^{-1}; periphyton: avg. 3.8 mg m^{-2}; min. 0.4 mg m^{-2}; max. 23.1 mg m^{-2}). Treatment effects were only occasionally observed as a significant increase in the chlorophyll-*a* biomass of the periphyton on day 14 (NOEC = 0.1 × EC_{50}), and a significant increase in the chlorophyll-*a* biomass of the phytoplankton on day 42 (NOEC = 0.1 × EC_{50}).

Species composition

The PRC plot (Figure 2) summarizes the response of the total phytoplankton community to herbicide application. The first PRC axis in this figure is plotted versus sampling time. In the RDA analysis, the total variance can be split up into three fractions: 22% is accounted for by the sampling date, 42% by treatment effects and 36% by variation between replicates. The horizontal axis of the PRC diagram displays the sampling date, while the vertical axis displays 21% of the variance explained by the treatment. Table 4 presents the results of the Monte Carlo permutation tests and the calculated $NOEC_{community}$ values, based on the Williams test. The PRC plot reveals that the species composition in all microcosms was relatively uniform in the pre-treatment period (day −7). After treatment with the herbicides the microcosms with the highest treatment level (1 × EC_{50}) immediately started to diverge from the other treatment levels. From day 14 onwards the 0.3 × EC_{50} treatment level showed a marked dissimilarity from the other treatments, though less pronounced than that at the 1 × EC_{50} treatment level, generally resulting in a $NOEC_{community}$ of 0.1 × EC_{50}. The single $NOEC_{community}$ of 0.03 × EC_{50} on day 42 was not taken into account, since this response did not follow the response incorporated in the Williams test. Towards the end of the experiment, the dissimilarity between the two highest treatment levels diminished. Species weights (b_k) of the taxa incorporated in the analysis are shown along side (Figure 2). These species weights can be interpreted as the affinity of the taxon with the PRC (Van den Brink and Ter Braak, acc.). Several taxa represented in Figure 3 (e.g., *Chlamydomonas* sp. (Figure 3A) and *Stephanodiscus/Cyclotella* sp. (Figure 3B)) have a positive species weight indicating an increase in abundance as a result of the herbicide application. *Monoraphidium* sp. (Figure 3D) and the filamentous blue-green algae were inhibited by the herbicide exposure. A large group of taxa, in the middle part of the list of species weights (Figure 2), showed no pronounced treatment effects.

Univariate analysis

Table 5 provides NOEC values for those phytoplankton taxa which showed a significant treatment effect after univariate testing by means of the Williams test. Most species with a high species weight in the PRC analysis also showed a significant effect after testing with the Williams test. In general, NOEC values were in agreement with the results from the PRC analysis, indicating effects at the 0.3 × EC_{50} treatment level and higher. Exceptions generally occurred on single occasions (e.g. Cyanophyta (day 35) with NOEC = 0.03 × EC_{50}, and the Chlorophyceae coccales with NOEC = 0.01 × EC_{50}). Just as in the PRC analysis, a distinction can be made between species which showed an increase in abundance and those which showed a decrease in abundance after herbicide application. Figure 3 illustrates the response of several phytoplankton taxa. *Chlamydomonas* sp. (3A) and *Stephanodiscus/Cyclotella* sp. (3B), were dominant taxa in the microcosms and showed an increase in abundance. The taxon *Stephanodiscus/Cyclotella* sp. showed two marked peaks on days 14 and 35 in both replicates at the two highest dosages. *Chlamydomonas* sp. displayed a gradual increase in abundance, resulting in a significant treatment effect from day 28 to day 42. On day 49, there was no significant treatment effect left and the abundance was approximately equal for all

Table 3. NOECs (No Observed Effect Concentration) per sampling date, calculated by the Williams test ($a \leq 0.05$), for the community metabolism endpoints. ($0.3 = 0.3 \times EC_{50}$)

Variable	Days post start application of herbicides								
	−7	3	7	14	21	28	35	42	49
Photosynthesis efficiency	–	0.3↓	0.1↓	–	0.3↓	–	–	–	
Dissolved oxygen	–	0.1↓	0.1↓	–	–	0.3↓	–	0.1↓	0.01↓
pH	–	0.1↓	0.01↓	–	–	–	0.3↓	–	–
Conductivity	–	–	–	–	–	–	0.3↑	0.3↑	0.3↑
Alkalinity	–	–	–	0.3↑	0.3↑	0.3↑	0.3↑	0.3↑	0.3↑
Inorganic nitrogen	–	–	0.3↑	0.1↑	–	0.3↑	0.3↑	–	–
Ortho-phosphate	–	–	–	–	–	–	–	–	–
Temperature	–	–	–	–	–	–	–	–	–

↑/↓, with increasing dosage of the herbicides a increase (↑) or decrease (↓) of the response was observed when compared with the control microcosms.
–, NOEC based on Williams test > maximal tested dosage ($1 \times EC_{50}$).

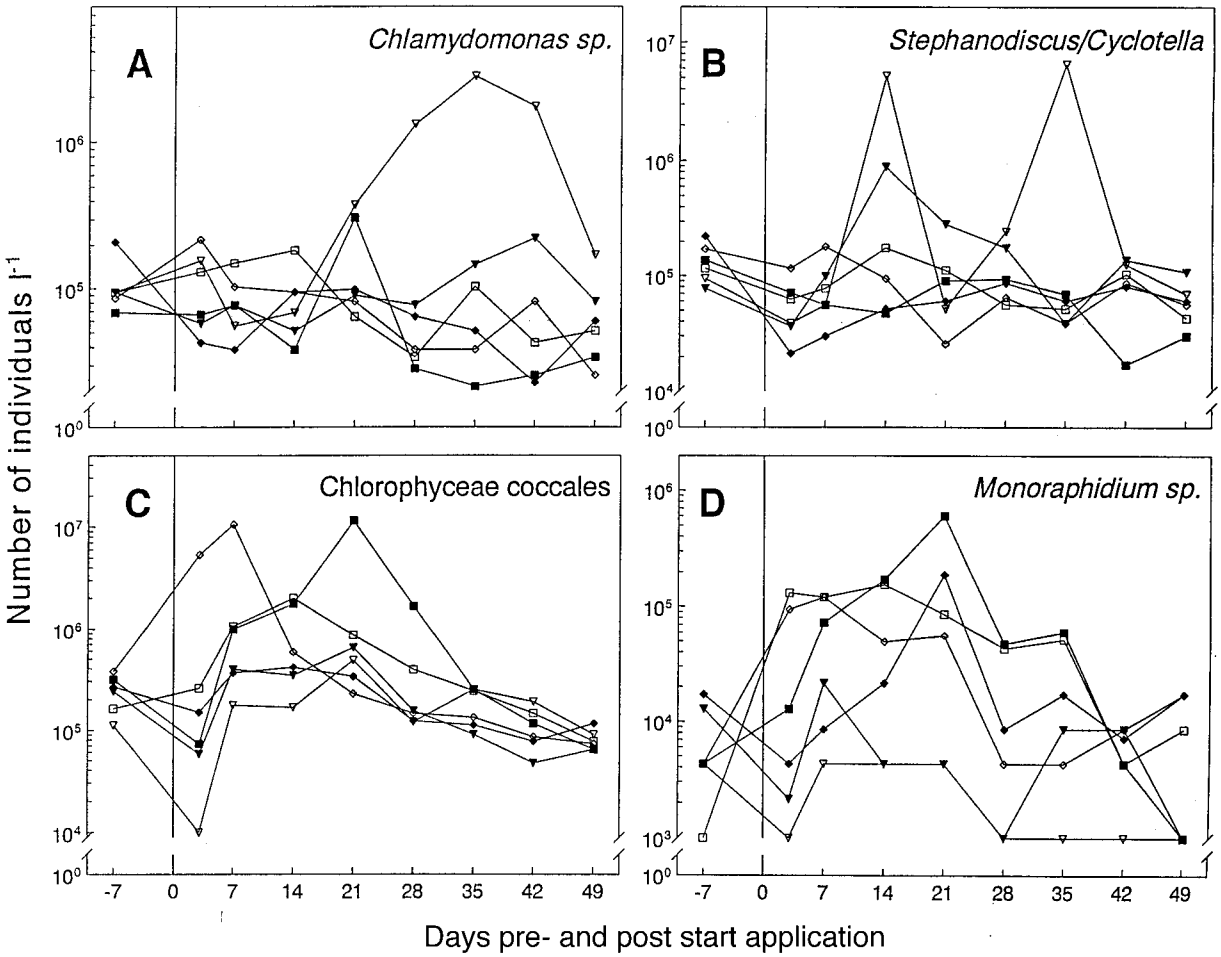

Figure 3. Dynamics (number of individuals l^{-1}) of four phytoplankton taxa expressed as the geometric means of the counted numbers per treatment level: *Chlamydomonas* sp. (A), *Stephanodiscus* sp./*Cyclotella* sp. (B), Chlorophyceae coccales (C) and *Monoraphidium* sp. (D). See for explanation symbols Figures 1 and 2.

Table 4. Results of the Monte Carlo permutation test (P-values) and calculated $NOEC_{community}$ values (Williams test, $a \leq 0.05$) for the phytoplankton and zooplankton community

Days post start application	Phytoplankton community		Zooplankton community	
	P-value	$NOEC_{community}$ (* EC_{50})	P-value	$NOEC_{community}$ (* EC_{50})
−7	>0.05	>1	>0.05	>1
3	>0.05	>1	>0.05	>1
7	>0.05	>1	>0.05	>1
14	0.003	0.1	>0.05	>1
21	0.003	0.1	>0.05	>1
28	0.027	>1	>0.05	>1
35	0.004	0.3	>0.05	>1
42	0.003	0.03	>0.05	>1
49	>0.05	>1	>0.05	>1

microcosms. *Monoraphidium* sp. (Figure 3D) showed a decrease in abundance at the highest two treatment levels. Week-to-week variability was high, while the abundance of this taxon was moderate. Based on the univariate analyses the Chlorophyceae coccales (Figure 3C), a small unidentified green alga, was the most sensitive taxon in the phytoplankton community, showing a significant decrease from day 14 to day 28 at treatment levels of 0.03 × EC50 and higher, resulting in a NOEC of 0.01 × EC50. Based on the multivariate and the univariate analysis of the phytoplankton community, the liberal $NOEC_{phytoplankton\ community}$ was 0.1 × EC_{50}. The NOEC of 0.01 × EC_{50} for the single algal taxon Chlorophyceae coccales should be viewed as a conservative approach.

Bacterioplankton

Data on bacterial activity in the microcosms, based on thymidine incorporation, cell numbers per litre, and specific growth rate, were tested with the Williams test. No consistently significant treatment effects could be observed for these variables throughout the experimental period.

Zooplankton community

At the start of the experiment (day −7), 20 different zooplankton taxa were observed. Within the first three weeks, 11 taxa were distinctly reduced in numbers in all microcosms, including the controls. The following five taxa were present in the microcosms throughout the experiment: *Keratella quadrata* (Rotatoria), *Daphnia galeata* (Cladocera), *Macrotrix laticornis* (Cladocera), Nauplius larvae and the cyclopoid copepods. The PRC plot (Figure 4) and the Monte Carlo permutation tests (Table 4) demonstrated that no significant treatment effect based on the total zooplankton community could be observed. Nevertheless, the zooplankton community at the highest three dosages ($\geq 0.1 \times EC_{50}$) seemed to deviate slightly from the control microcosms on days 35 and 42. The species weights (b_k) displayed alongside Figure 4 indicate that this was caused by the taxa *Daphnia galeata* and cyclopoid copepods, both of which occurred in low abundance throughout the experiment. Figure 5 presents the abundance of *Daphnia galeata* and cyclopoid copepods for the sampling dates on which significant treatment effects (Williams test) could be observed ($NOEC_{cyclopoid\ copepods} < 0.01 \times EC_{50}$ (day 35) and $NOEC_{cyclopoid\ copepods} = 0.03 \times EC_{50}$ (day 42); $NOEC_{Daphnia\ galeata} = 0.03 \times EC_{50}$ (day 28 and 35). In this period, densities of these taxa were always less than 6 individuals l^{-1}. Although the multivariate and univariate analysis revealed a trend in effects on the zooplankton community, this trend was qualitatively and quantitatively small. The NOEC of 0.03 × EC_{50} for *Daphnia galeata*, which was found at two consecutive sampling dates, should therefore be regarded as conservative. The NOEC values for the cyclopoid copepods seemed to vary with the sampling dates, and considering the minute effect (Figure 5) this taxon was not included in the hazard assessment.

Table 5. NOECs per sampling date, calculated by the Williams test ($a \leq 0.05$), for the abundances of the most abundant and sensitive phytoplankton taxa which show a significant treatment effect and the general taxonomic groups. ($0.3 = 0.3 \times EC_{50}$)

Taxon	Days post start application of herbicides								
	−7	3	7	14	21	28	35	42	49
Baciyllariophyceae	–	–	–	0.3↑	–	–	0.3↑	–	–
Stephanodiscus/Cyclotella	–	–	–	0.1↑	–	–	0.3↑	–	–
Chlorophyceae	–	–	–	0.01↓	–	–	0.3↑	0.3↑	–
Chlamydomonas sp.	–	–	–	–	–	0.3↑	0.1↑	0.3↑	–
Monoraphidium sp.	–	–	–	0.1↓	0.1↓	–	–	–	–
Chlorophyceae coccales	–	–	–	0.01↓	0.01↓	0.01↓	–	–	–
Cryptophyceae	–	0.3↓	–	–	–	–	–	–	0.3↑
Cyanophyta	–	–	–	–	–	–	0.03↓	0.3↓	–
Cyanophyta filaments	–	–	–	–	0.3↓	–	0.03↓	–	–

↑/↓, with increasing dosage an increase in abundance (↑) or decrease in abundance (↓) was observed when compared with the control microcosms.
–, NOEC based on Williams test > maximal tested dosage ($1 \times EC_{50}$)

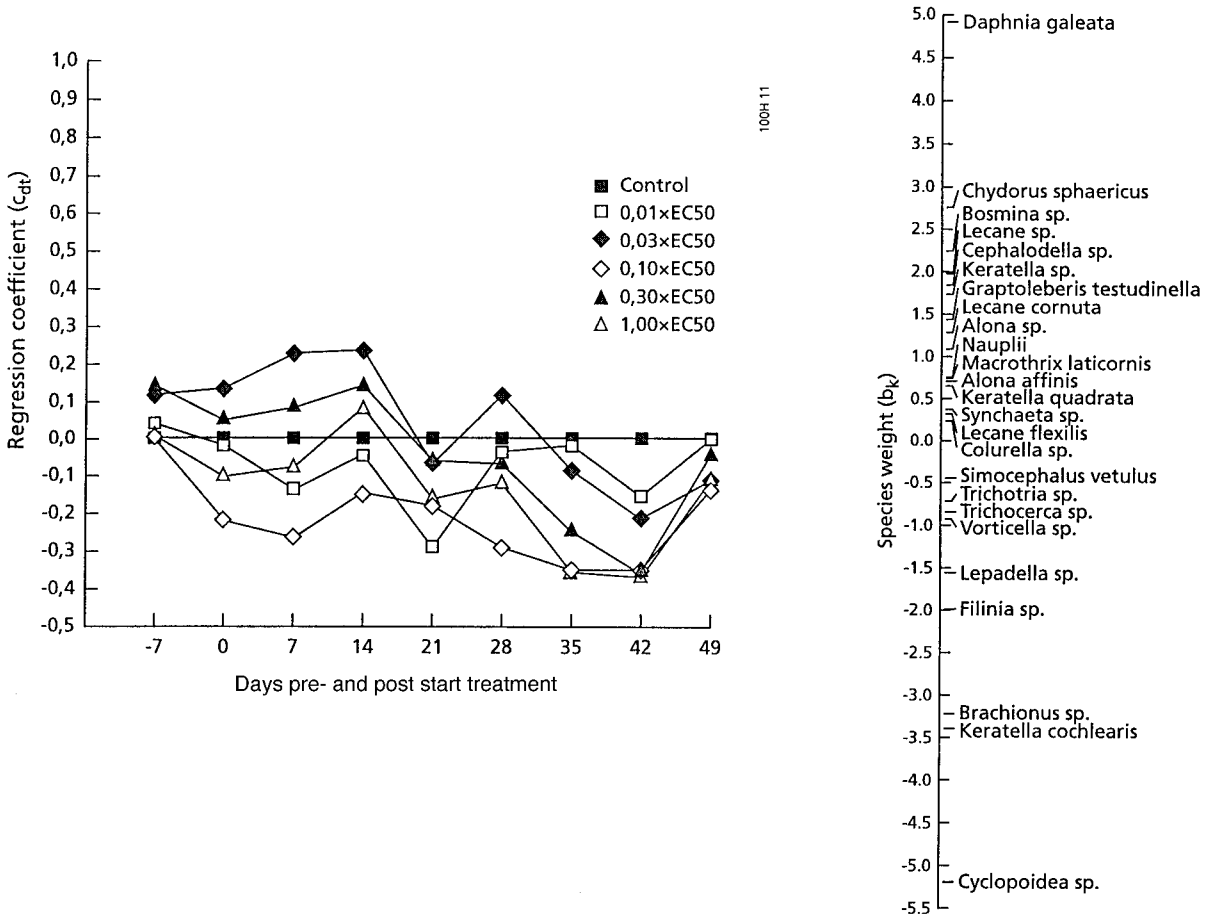

Figure 4. Principle Response Curve (PRC) of zooplankton community. The deviance of each of the treatment levels from the control is depicted. The species weights, shown on the side of the plot, indicate the affinity of each individual taxon with the PRC. A negative species weight indicates that the abundance of the species increased towards the end of the experiment in the highest treatments.

Macro-invertebrates

At the start of the experiment (week −1), 44 different macro-invertebrate taxa were observed, with the individuals evenly distributed among the microcosms. Characteristic taxa included: *Asellus aquaticus*, *Echinogammarus ischnus*, *Dugesia lugubris/polychroa*, *Bithynia tentaculata* and *B. leachi*. At the end of the experiment (week 8), 37 different macro-invertebrate taxa could be observed. The total number of individuals had decreased slightly. None of the taxa showed a significant decrease or increase with increasing herbicide dosage when tested with the Williams test.

Discussion

Fate of herbicides

In our study, explaining the $t_{1/2}$ being concentration dependent is difficult, since no information is present on how much active ingredient was applied in total, processes of sorption and desorption etc. The phenomenon, however, was also found by Van den Brink et al. (1997) for the herbicide linuron. Linuron caused inhibition of the primary production, resulting, e.g. in a decreased pH followed by a slower hydrolysis of the herbicide. In our experiment, a decreased pH also occurred at the highest treatment levels, but the decrease was much smaller than in the experiment with linuron (pH drop from 8.7 to 8.1 and from 9.9 to 7.8 respectively). Furthermore, the pH decrease in our experiment occurred only at the highest two treatment levels, whereas the dose-dependent decay rate increased from the lowest treatment levels onwards. Khan in Solomon et al. (1996) observed a pH dependent decay rate for atrazine, calculated according to first order kinetics. Comparable results for diuron and metolachlor are not available.

Ecological effects

The following effects were observed (Figure 6). (1) Direct effects became apparent from an initial drop in photosynthesis efficiency, pH and oxygen concentration and a decrease in the abundance of several phytoplankton taxa. (2) 14 days post application, an increase in the abundance of several phytoplankton taxa (*Chlamydomonas* sp. and *Stephanodiscus/Cyclotella)* was observed; oxygen concentrations recovered while alkalinity, conductivity and total inorganic nitrogen were elevated. (3) Effects on fauna were small; *Daphnia galeata* showed a decreasing trend while the cyclopoid copepods tended to increase at the end of the experiment. The analyses of the total zooplankton and macro-invertebrate communities as well as that of the bacterioplankton in the waterphase revealed no significant treatment effects.

The treatment effects observed for the community metabolism endpoints largely resulted from effects on the phytoplankton community. The initial drop in photosynthesis efficiency, oxygen concentration and pH can be attributed to the direct inhibitory effects of the herbicides on the phytoplankton. These effects could no longer be observed once *Stephanodiscus/Cyclotella* and *Chlamydomonas* sp. started to bloom. Raised concentrations of inorganic nitrogen were found at the highest treatment levels following the initial decrease in abundance of several phytoplankton taxa. This indicates that either the available nitrogen could not be used, or that additional nitrogen was released via degradation of the algae, although treatment effects on the bacterioplankton could not be demonstrated. Alkalinity and conductivity, which are strongly correlated at low nutrient levels, showed a positive treatment effect from, respectively, day 14 and day 35 onwards. These variables indicate changes in the functioning of the ecosystem. The interpretation of changes in alkalinity or conductivity in terms of changes in the pool of inorganic carbon or nutrients is, however, complicated (Kersting, 1994).

The algal taxa which showed a decrease in growth with increasing dosage were probably directly affected by inhibition of photosynthesis due to the herbicide application. *Stephanodiscus/Cyclotella* (Figure 3B) and *Chlamydomonas* sp. (Figure 3A) showed a marked increase in abundance with increasing dosage. Ietswaart (pers. comm.) found that *Stephanodiscus/Cyclotella* is less sensitive to the selected herbicides ($EC_{50,\ 72h\ atrazine} = 657\ \mu g\ l^{-1}$; $EC_{50,\ 72h\ diuron} = 27\ \mu g\ l^{-1}$; $EC_{50,\ 72h\ metolachlor} = 485\ \mu g\ l^{-1}$) than *S. capricornutum*. The increased biomass of this taxon was probably caused by an indirect effect, such as a release of nutrients by the decay of more susceptible algae, reduced competition or reduced grazing pressure. The abundance of *Stephanodiscus/Cyclotella* showed two peaks (day 14 and day 35). Although this effect did not occur on two consecutive sampling dates, its occurrence was so obvious in both replicates that this effect was regarded as a treatment effect. On the sampling dates between those displaying high abundance, biomass returned to control levels. This return

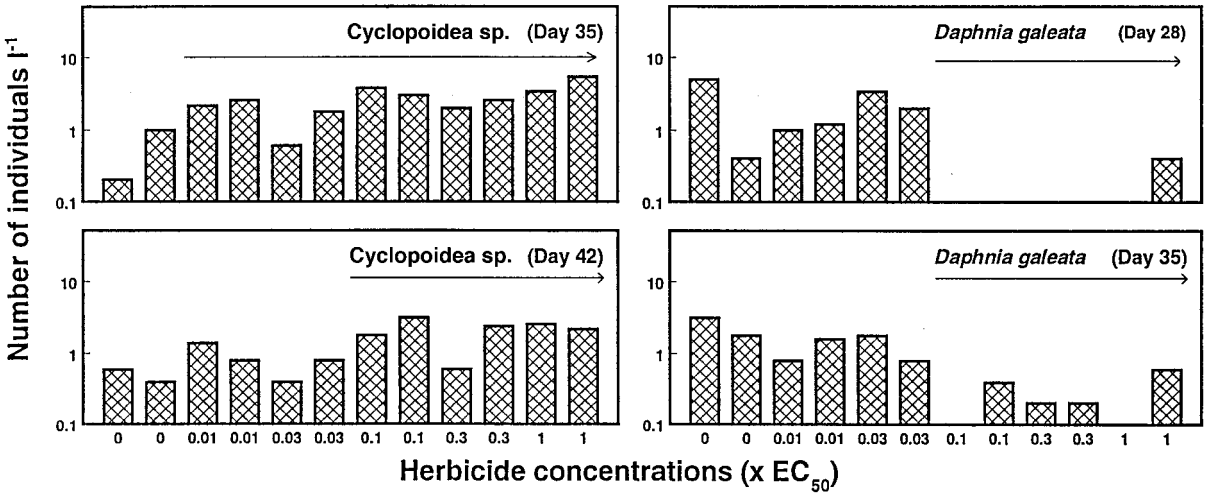

Figure 5. Dynamics (number of individuals l^{-1}) of two zooplankton taxa: cyclopoid copepods (day 35 and 42) and *Daphnia galeata* (day 28 and 35). The abundances are shown for each individual cosm. Arrow indicates from which herbicide concentration onwards a significant treatment effect is observed based on analysis with the Williams test ($a \leq 0.05$).

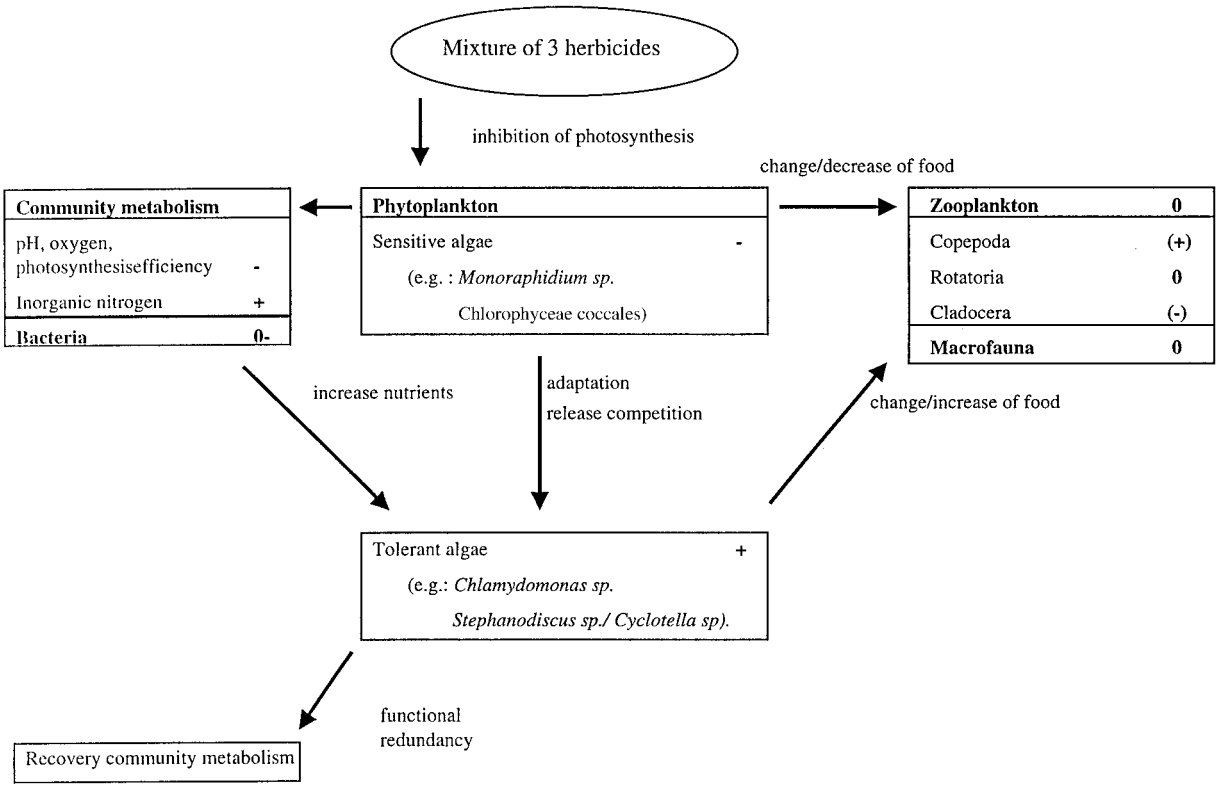

Figure 6. Illustration of the effect chain in the microcosms following a chronic application of a mixture of three herbicides (atrazine, diuron and metolachlor).

could be caused by e.g. a lack of an element essential for the growth of the algae, which was then released after the decay of the algae. A fungal infection of the cells, like that observed by Lund and Canter-Lund (1995) and by Van Donk and Ringelberg (1983) could not be demonstrated, even after careful observation. The temperature on day 28 was increased by 2–3 °C due to a temporary malfunction of the cooling installation, which could also have affected the abundance of *Stephanodiscus/Cyclotella*. This temperature effect was, however, not present on day 42, when the abundance once again returned to control levels.

The sensitivity of *Chlamydomonas* sp. found in standard toxicity tests ($EC_{50,\ 72\ h\ atrazine} = 98\ \mu g\ l^{-1}$; $EC_{50,\ 72\ h\ diuron} = 32\ \mu g\ l^{-1}$; $EC_{50,72hmetolachlor} = 87\ \mu g\ l^{-1}$) does not differ much from that of *S. capricornutum*. Hence, the increased biomass of this taxon might be explained by its ability to adapt to herbicides, combined with the observed increased nitrogen levels and constant or slightly reduced zooplankton grazing pressure. Several authors have reported adaptation of algae after herbicide application, although this effect is not frequently observed in microcosm experiments. Van den Brink et al. (1997) performed a microcosm experiment with a chronic low-level application of linuron and observed a bloom of *Chlamydomonas* sp. They also proved adaptation in a lab experiment with *Chlamydomonas reinhardtii*. Kasai & Hanazato (1995) observed enhanced tolerance to simetryn of *Scenedesmus* isolated from ponds treated previously with this herbicide. Increased tolerance to diuron and atrazine has been observed for marine periphyton communities (Molander & Blanck, 1992) and freshwater phytoplankton communities (DeNoyelles et al., 1989) respectively.

The indications of an increase in algal biomass from the physical/chemical measurements at the highest treatment levels were not confirmed by the chlorophyll-*a* analyses. This does not mean, however, that algal biomass was not elevated. Chlorophyll-*a* estimation forms a crude description of the phytoplankton population because no distinction is made between the different taxa. Furthermore, one should be aware that pigment concentrations in algae can vary greatly depending on taxonomic group, metabolism, light, temperature, nutrient availability and many other factors (Wetzel & Likens, 1991).

Effects on the zooplankton community were scarcely observed. On the basis of the PRC analysis of the total zooplankton community, no significant treatment effects could be established. *Daphnia galeata* was the only species for which a quantitatively small but significant treatment effect could be demonstrated for two consecutive sampling dates (day 28 and 35); there was a decrease in abundance in the post-treatment period. This decrease was most probably not caused by direct effects of the herbicides (Figure 7), and might be explained by a change in the food source or a change in the competition for a food source. The 'competition for food' theory is supported by the fact that cyclopoid copepods showed a minor increase in abundance in this period. On the other hand, *Chlamydomonas* sp, which generally provides a good food source for both taxa (e.g., Sterner, 1989) showed high abundances in these weeks. Since both taxa were not abundant within the microcosms (abundance <6 individuals l^{-1} on the sampling dates presented), pronounced changes in grazing pressure on the algae could not have occurred.

Comparison with other studies

Based on the reviews by Brock & Budde (1994) and Kersting (1994), the effects observed in this experiment can be regarded as comparable to those found in other ecosystem studies with individual herbicides. Systems treated with herbicides generally show a decrease in the functioning of primary producers, followed by a decrease in dissolved oxygen and pH levels. Changes in the abundance of algae or macrophytes can often be observed, while changes in alkalinity and/or conductivity are often found to be the opposite of those for dissolved oxygen or pH.

Figure 7 presents an overview of literature data derived from single species experiments and micro- or mesocosm studies of individual herbicides, in comparison with the results of our study. The axes are scaled according to the EC_{50} values of *S. capricornutum*, the most sensitive standard test organism for the substances tested. Effects of atrazine have been studied extensively and were recently reviewed by Solomon et al. (1996). Based on more than 20 micro- and mesocosm studies they conclude that the $NOEC_{ecosystem,\ atrazine} = 20\ \mu g\ l^{-1}$. They state that, although the structure of the aquatic community may change at lower concentrations, the functions of the species affected are taken over (functional redundancy). Nevertheless, Van den Brink et al. (1995) reported a slight trend towards short-term decrease in the photosynthetic activity during a 50 days chronic application of 5 $\mu g\ l^{-1}$ atrazine. They did not observe significant effects on plankton community structure.

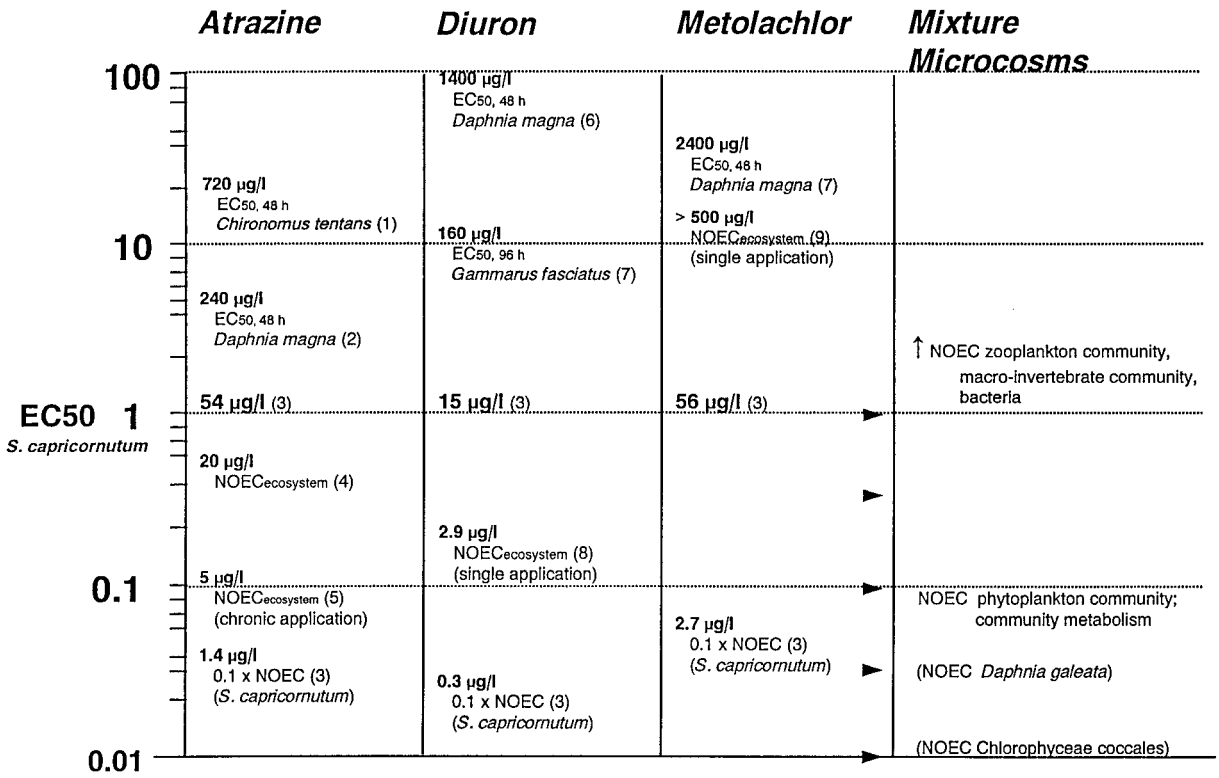

Figure 7. Comparison of literature based information of effects of the individual herbicides and the information obtained from the microcosm experiment concerning a mixture of the herbicides. The values are scaled to the EC_{50} values of the most susceptible standard test organism: *Selenastrum capricornutum.* ▶: tested concentration in the cosm-experiment. Literature references: (1) Macek, 1976; (2) Pott, 1980; (3) Ietswaart (pers. comm.); (4) Solomon et al, 1996; (5) Van den Brink et al., 1995; (6) Crommentuijn et al., 1997 (7) Flum & Shannon, 1987; (8), Huber (pers. comm.).

The only freshwater ecosystem study of diuron known to us was performed by Flum & Shannon (1987). In this study, the pH of the overlying water was the most sensitive endpoint, which is in accordance with results of our study. A NOEC of 2.9 $\mu g\ l^{-1}$ and a LOEC of 28.5 $\mu g\ l^{-1}$ were observed for this endpoint.

The only freshwater microcosm study of metolachlor known to us was performed by Huber (pers. comm.). In this study, single applications of 5 to 500 $\mu g\ l^{-1}$ did not lead to any treatment effect in the functioning or structure of the microcosm. Unlike the results of e.g. atrazine and diuron the ecosystem effects of metolachlor found by Huber (pers. comm.) seem to be less grave than could be expected on the basis of single species experiments. The study did not find a dose dependent degradation of the herbicides, which is in contrast with the results of our study.

Our study, with a mixture of herbicides cannot be compared directly with studies using individual substances. Therefore, an answer to the question whether or not the herbicides are additive when applied simultaneously, cannot really be given. Faust et al. (1993) assessed the toxicity of 29 binary mixtures of 9 herbicides with different modes of action on the green alga *Chlorella fusca* and found that the assumption of concentration addition was valid for 85% of the mixtures. The fact that the majority of the effects in our microcosm experiment resulted in a NOEC of 0.1 $\times$ EC_{50} indicates that the safe threshold levels of the mixture are not much lower than the $NOEC_{ecosystem}$ of the most toxic individual compound (Figure 7). The effects found in our study do not seem to contradict concentration addition of the herbicides.

Data analysis

The results of the multivariate analysis at the community level and the results of the univariate analysis are, to a large extent, in agreement with each other. We set the criterium that positive results of the Williams test should occur on at least two consecutive sampling

weeks. We are well aware that, by leaving incidental NOECs out of consideration, small transient effects on these taxa may be overlooked. The risk of a type I error is, however, greatly diminished. The Williams test assumes a monotonic increase or decrease of the response with increasing dose. If the data show other patterns, the outcome of the test is not reliable.

Conclusions

In the present study, chronic exposure to a mixture of the herbicides atrazine, diuron and metolachlor resulted in a shift in the phytoplankton community and a temporary disturbance of the community metabolism. The primary producers show pronounced effects as a direct consequence of the application of the herbicides. This resulted in a NOEC for the phytoplankton community and community metabolism of 0.1 $\times$ EC_{50}. The NOEC for the zooplankton community, macro-invertebrates and bacteria was $\geq 1 \times EC_{50}$.

Nevertheless, one representative of the zooplankters (*Daphnia galeata*) showed a small but significant decrease in abundance, resulting in a conservative NOEC of 0.03 $\times$ EC_{50}. A similar conservative estimation resulted in a NOEC of 0.01 $\times$ EC_{50} for the most sensitive phytoplankton taxon, Chlorophyceae coccales. Although this effect was statistically significant, the magnitude of the effect was moderate (Figure 3C). This was also reflected by a relatively small species-weight in the PRC analysis. In this conservative approach, the $NOEC_{\text{Chlorophyceae coccales}}$ was slightly lower than the appropriate safety factor proposed by the Uniform Principles, in this case 0.1 $\times$ $NOEC_{S.capricornutum,\ \text{diuron}}$, and therefore would not provide adequate protection of this taxon.

All other observed variables in this aquatic ecosystem were sufficiently protected against the mixture by the safety factors proposed in the Uniform Principles for single compounds (Figure 7). In the natural environment, the effects of herbicide application would most likely be less grave than in our indoor microcosm study, thus justifying this liberal approach. Species would be able to recolonize from upstream areas, and structural changes would thus be short-lived in natural systems.

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