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

I. Primary Producers

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

INTRODUCTION

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

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

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

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

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

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

MATERIALS AND METHODS

Experimental Design

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

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

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

Linuron Application and Analysis

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

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

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

Endpoints

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

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

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

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

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

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

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

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

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

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

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

Bioassay with the Macrophyte Species Elodea nuttallii

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

Laboratory Test Performed with Chlamydomonas reinhardtii

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

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

Data Analysis

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

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

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

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

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

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

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

RESULTS

Linuron Concentrations

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

Bioassay and Final Harvest Elodea nuttallii

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

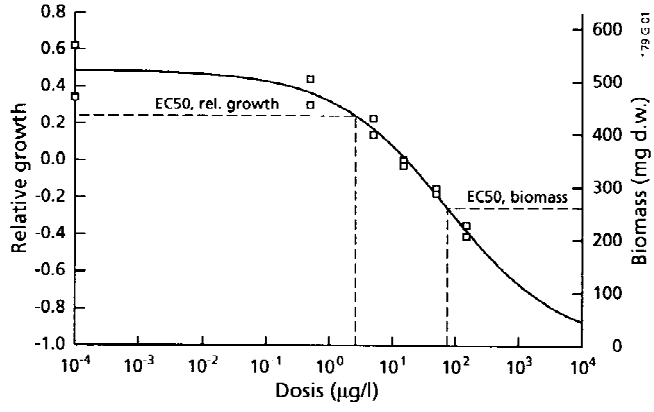

FIG. 1. The measured biomass and relative growth of *Elodea nuttallii* in the bioassay incubated in the microcosms and the model for EC_{50} calculations.

TABLE 2

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

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

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

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

Phytoplankton

The RDA-biplot (Fig. 3) can be seen as a summary of the total phytoplankton data set. Most of the variation expressed on the first axis can be attributed to changes in species composition over time, those expressed on the second axis to the treatment. In the diagram, samples with nearly identical species composition lie close together, while samples with very different species composition lie far apart. If an imaginary line is drawn through a species point and the origin of the plot, the relative abundance of this species in all samples can be derived by perpendicularly projecting the sample points on this imaginary line. The samples whose projection on the ''species line'' is far away from the origin, but on the same side of the origin as the species point, contain relatively high numbers of this species. The greater the distance between the projection of a sample and the origin, the more abundant this species is in that sample. If a sample point projects on the other side of the origin, compared to the species point, numbers of this species are relatively low in that sample. In the diagram, the taxon *Cocconeis* sp. is relatively abundant in all control samples and (almost) absent from the samples at the highest treatment level. To limit the number of taxa presented in the diagram, only the 13 taxa most discriminant for the analysis have been included.

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

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

Periphyton and Neuston

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

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

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

Chlorophyll-a

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

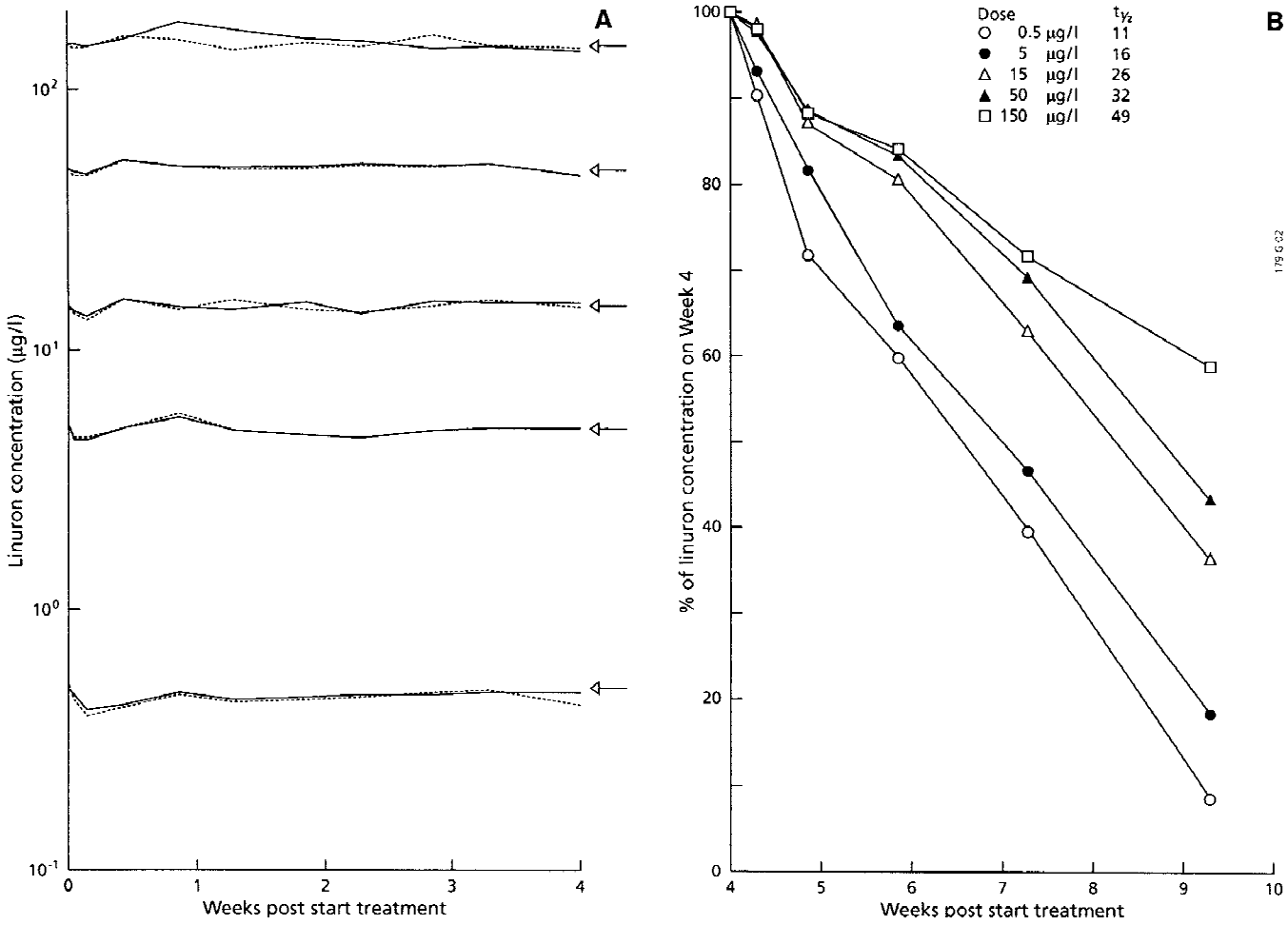

FIG. 2. Dynamics in linuron concentration per dose. (A) The linuron concentration per replicate in time for the treatment period. The arrows represent the target concentrations. (B) The decrease in linuron concentration per treatment level in the posttreatment period as a percentage of the actual linuron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

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

Laboratory Test with Chlamydomonas reinhardtii

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

DISCUSSION

Fate of Linuron

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

TABLE 3

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

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

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

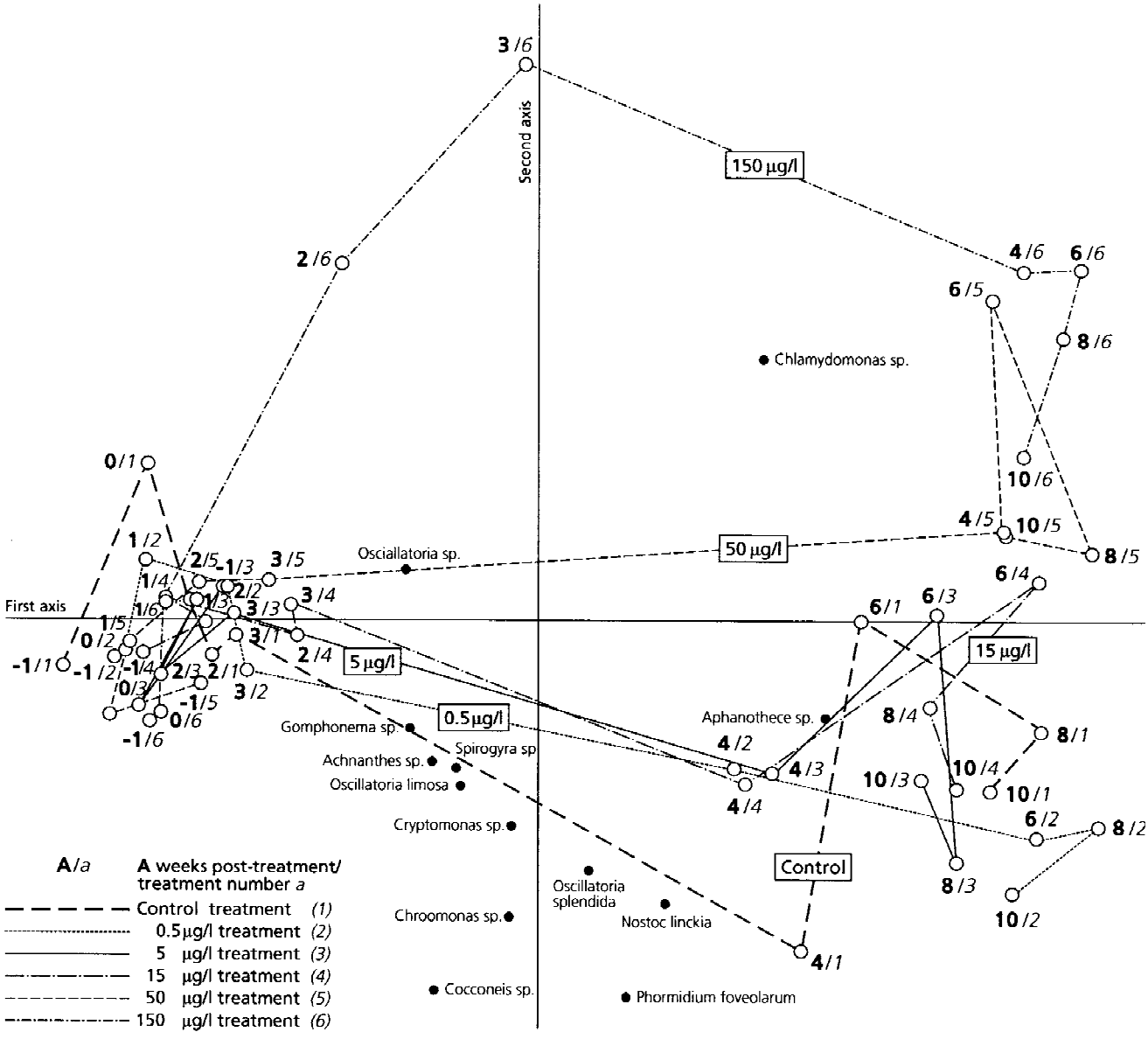

FIG. 3. Ordination diagram (RDA) indicating effects of the herbicide linuron on the phytoplankton per treatment level. Sampling date and treatment level, and their interactions, were taken as explanatory variables. The first number of the labels of the sample-points refer to the sampling week, the second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

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

Effects on Elodea nuttallii

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

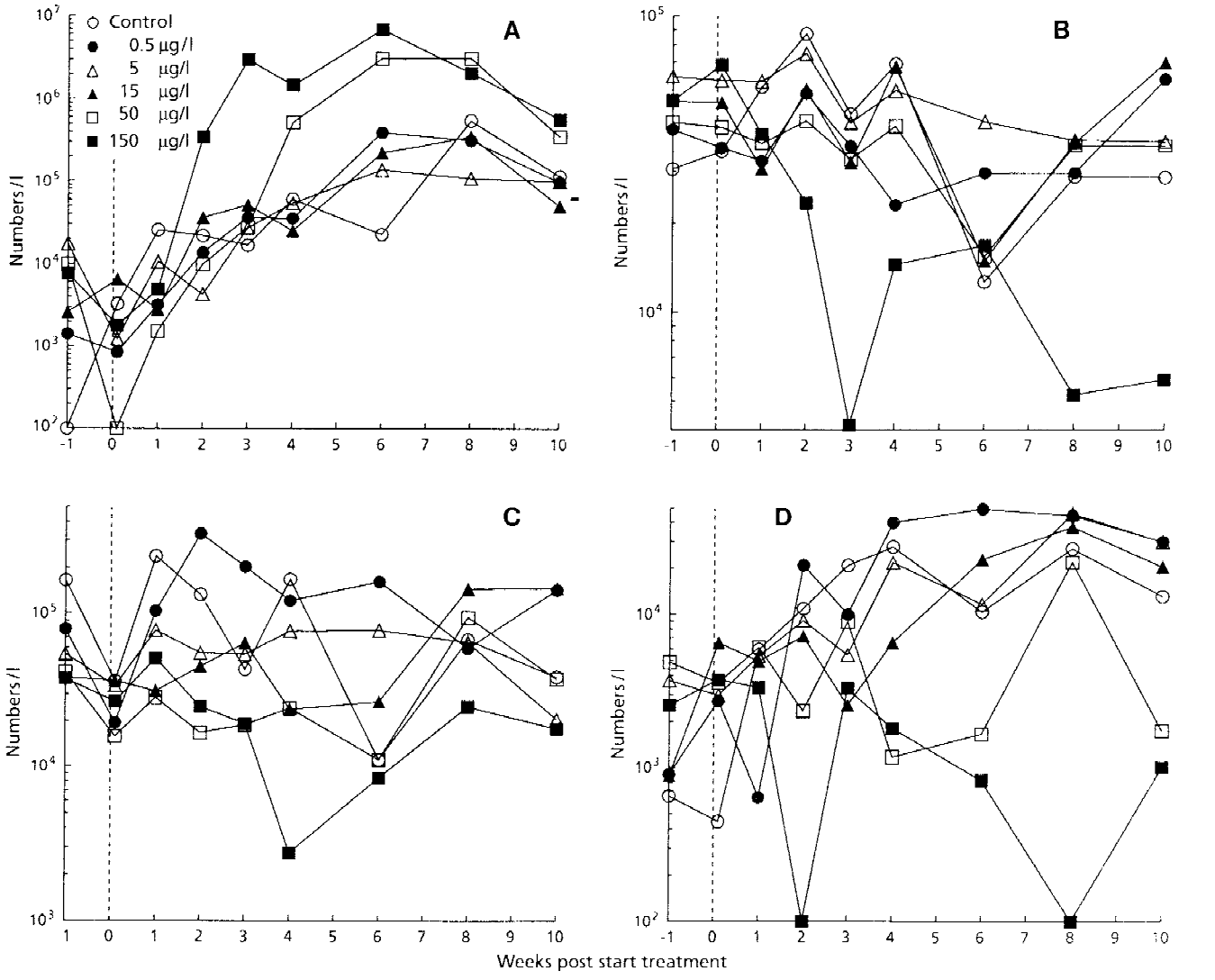

FIG. 4. Dynamics in numbers of four phytoplankton taxa. (A–D) The geometric means of the counted numbers per treatment level, of *Chlamydomonas* (A), *Cocconeis* (B), *Chroomonas* (C), and *Phormidium foveolarum* (D), are shown.

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

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

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

TABLE 4

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

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

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

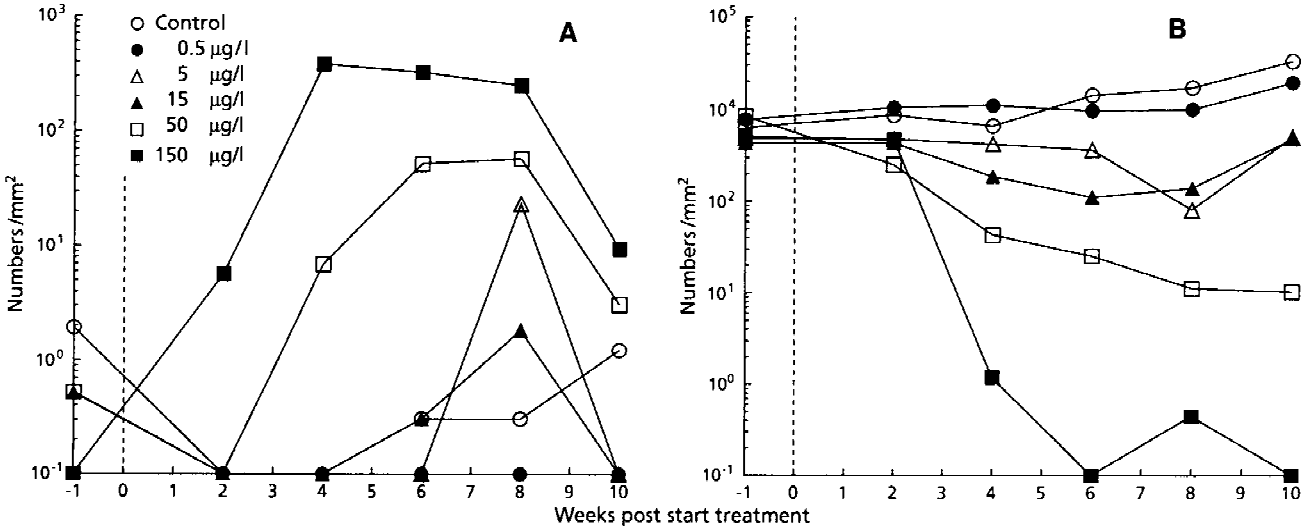

FIG. 5. Dynamics in numbers of two periphyton taxa on glass slides. (A and B) The geometric means of the counted numbers per treatment level of *Chlamydomonas* sp. (A) and *Cocconeis* sp. (B) are shown.

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

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

Effects on Algae

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

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

TABLE 5

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

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

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

Comparison of Results with Other Micro/Mesocosm Studies

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

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

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

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

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

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

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

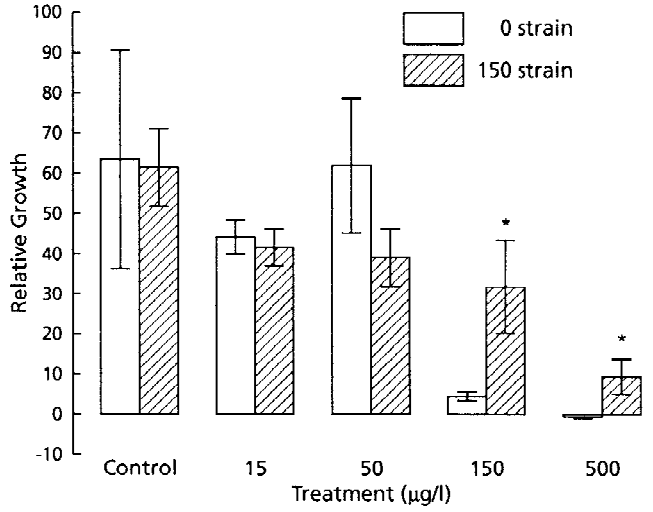

FIG. 6. Relative growth of two strains of *Chlamydomonas reinhardtii*, one strain originated from a linuron-free medium and one strain from a culture previously treated with 150 μg/L linuron. The two strains were exposed to 0, 15, 50, 150, and 500 μg/L linuron for 3 days. Significant differences between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

Hazard Assessment

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

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

CONCLUSIONS

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

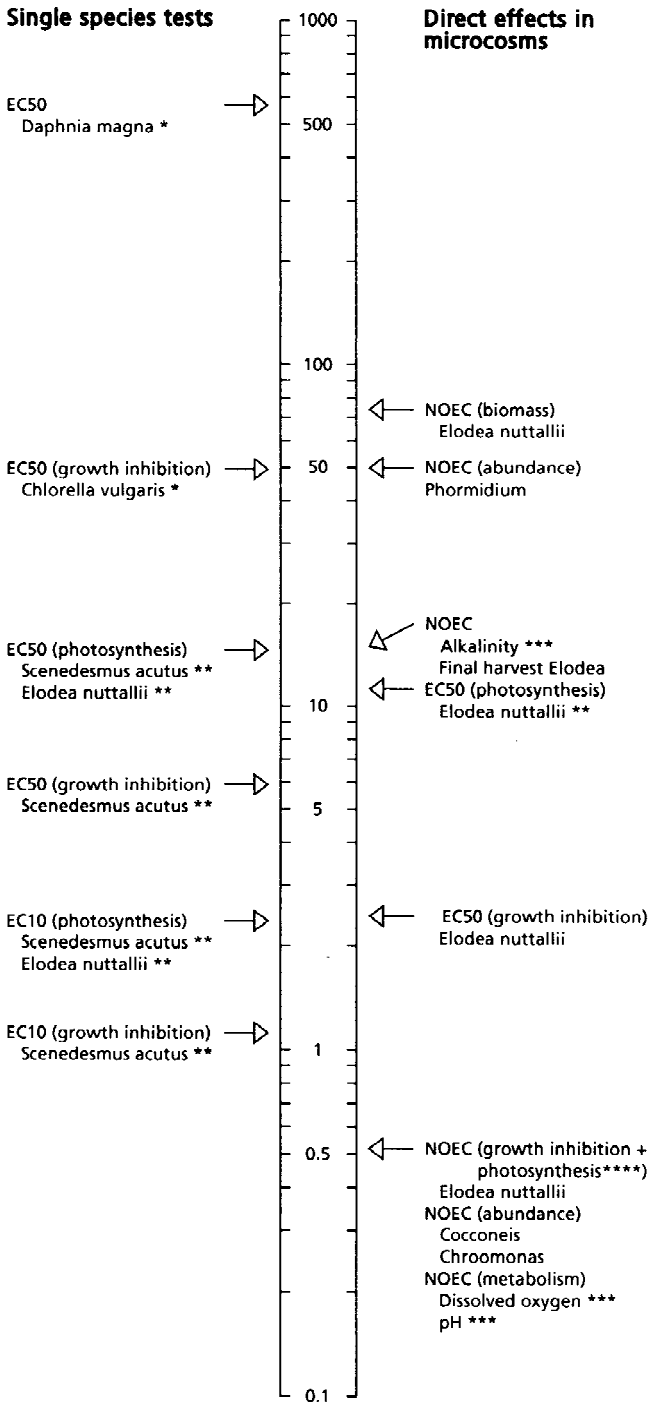

FIG. 7. Summary of laboratory tests results with linuron and direct effects found in the microcosms. *Stephenson and Kane, 1984; **Snel *et al.*, submitted; ***Cuppen *et al.*, submitted; ****Own calculations (Williams test, $P \leq 0.05$) on data from Snel *et al.*, submitted.

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

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REFERENCES

Brock, T. C. M., and Budde, B. J. (1994). On the choice of structural parameters and endpoints to indicate responses of freshwater ecosystems to pesticide stress. In *Freshwater Field Tests for Hazard Assessment of Chemicals* (I. A. Hill, F. Heimbach, P. Leeuwangh, and P. Matthiesen, Eds.), pp. 19–56. Lewis Publishers, Boca Raton, FL.

Brock, T. C. M., Crum, S. J. H., Van Wijngaarden, R., Budde, B. J., Tijink, J., Zuppelli, A., and Leeuwangh, P. (1992). Fate and effects of the insecticide Dursban 4E in indoor *Elodea*-dominated and macrophyte-free freshwater model ecosystems: I Fate and primary effects of the active ingredient chlorpyrifos. *Arch. Environ. Contam. Toxicol.* **23,** 69–84.

Brock, T. C. M., Roijackers, R. M. M., Rollon, R., Bransen, F., and Van der Heyden, L. (1995). Effects of nutrient loading and insecticide application on the ecology of *Elodea*-dominated freshwater microcosms. II. Responses of macrophytes, periphyton and macroinvertebrate grazers. *Arch. Hydrobiol.* **134,** 53–74.

Brockway, D. L., Smith, P. D., and Stancil, F. E. (1984). Fate and effects of atrazine in small aquatic microcosms. *Bull. Environ. Contam. Toxicol.* **32,** 345–353.

Cairns, J. (1986). The myth of the most sensitive species. *Bioscience* **36,** 670–672.

Cserhati, T., Vegh, A., and Dobrovolszky, A. (1976). Study on the factors influencing the decomposition of linuron. I. The temperature and pH dependence of the linuron decomposition. *Acta Phytopathologica Academiae Scie. Hung.* **11,** 325–330.

Cuppen, J. G. M., Van den Brink, P. J., Van der Woude, H., Zwaardemaker, N., and Brock, T. C. M. Sensitivity of macrophyte-dominated freshwater microcosms to chronic levels of the herbicide linuron. II. Invertebrates and community metabolism. Submitted.

DeNoyelles, F., Kettle, W. D., Fromm, C. H., Moffett, M. F., and Dewey, S. L. (1989). Use of experimental ponds to assess the effects of a pesticide on the aquatic environment. *Misc. Pub. Ent. Soc. Am.* **75,** 41–56.

Draxl, R., Neugebaur, K. E., Zieris, F. J., and Huber, W. (1991). Comparison of the ecological effects of diquat on laboratory multi-species and outdoor freshwater systems. *Verh. Internat. Verein. Limnol.* **24,** 2269–2271.

EU (1994). Council Directive 94/43/EC of 27 july 1994; Establishing Annex IV to Directive 91/414/EEC Concerning the Placing of Plant Protection Products on the Market. *Off. J. Eur. Commun.* **L 227,** 31–55.

Giddings, J. M. (1980). Types of aquatic microcosms and their research applications. In *Microcosms in Ecological Research* (J. P. Giesy, Ed.), pp 248–266. DOE Symposium Series 52, Technical Information Centre, U.S. Department of Energy.

Hodgson, L. M., and Linda, S. B. (1984). Response of periphyton and phytoplankton to chemical control of *Hydrilla* in artificial pools. *J. Aquat. Plant Managem.* **22,** 48–52.

Hommen, U., Veith, D., and Ratte, H. T. (1994). A computer program to evaluate plankton data from freshwater field tests. In *Freshwater Field Tests for Hazard Assessment of Chemicals* (I. A. Hill, F. Heimbach, P. Leeuwangh and P. Matthiesen, Eds.), pp. 503–513. Lewis Publishers. Boca Raton, FL.

Jenkins, D. G., and Buikema, A. L. (1990). Response of a winter plankton food web to simazine. *Environ. Toxicol. Chem.* **9,** 693–705.

Jüttner, I., Peither, A., Lay, J. P., Kettrup, A., and Ormerod, S. J. (1995). An

outdoor mesocosm study to assess ecotoxicological effects of atrazine on a natural plankton community. *Arch. Environ. Contam. Toxicol.* **29,** 435–441.

Kasai, F., and Hanazato, T. (1995) Genetic changes in phytoplankton communities exposed to the herbicide simetryn in outdoor experimental ponds. *Arch. Environ. Contam. Toxicol.* **28,** 154–160.

Kemp, W. M., Boynton, W. R., Cunningham, J. J., Stevenson, J. C., Jones, T. W., and Means, J. C. (1985). Effects of atrazine and linuron on photosynthesis and growth of the macrophytes *Potamogeton perfoliatus* L. and *Myriophyllum spicatum* L. in an estuarine environment. *Marine Env. Res.* **16,** 255–280.

Kersting, K. (1994). Functional endpoints in field testing. In *Freshwater Field Tests for Hazard Assessment of Chemicals* (I. A. Hill, F. Heimbach, P. Leeuwangh, and P. Matthiesen, Eds.), pp. 57–81. Lewis Publishers, Boca Raton, FL.

Kimball, K. D., and Levin, S. A. (1985). Limitations of laboratory bioassays: the need for ecosystem-level testing. *Bioscience* **3,** 165–171.

La Point, T. W., and Perry, J. A. (1989). Use of experimental ecosystems in regulatory decision making. *Env. Managem.* **13,** 539–544.

Lynch, T. R., Johnson, H. E., and Adams, W. J. (1985). Impact of atrazine and hexachlorobiphenyl on the structure and function of model stream parameters. *Environ. Toxicol. Chem.* **4,** 399–413.

Moed, J. R., and Hallegraeff, G. M. (1978). Some problems in the estimation of chlorophyll-a and phaeopigments from pre- and post-acidification spectrophotometric measurements. *Int. Revue Ges. Hydrobiol.* **63,** 787–800.

Molander, S., and Blanck, H. (1992). Detection of pollution-induced community tolerance (PICT) in marine periphyton communities established under diuron exposure. *Aquat. Toxicol.* **22,** 129–144.

Paterson, D. M., and Wright, S. J. L. (1987) A continuous-flow model ecosystem for studying effects of herbicides on aquatic plants. *Weed Sci.* **35,** 704–710.

Payne, R. W., and Lane, P. W. (1987). *Genstat 5,* Reference manual. Clarendon Press, Oxford, UK.

Peichl, L., Lay, J. P., and Korte F. (1985). Wirkung von Atrazin und 2,4 Dichlorphenoxyessigsäure auf die Populationsdichte von Phyto- und Zooplankton in einem aquatischen Freilandsystem. *Zeitschr. Wass. Abwass. Forsch.* **18,** 217–222.

Persoone, G., and Janssen, C. R. (1994). Field validation based on laboratory tests. In *Freshwater Field Tests for Hazard Assessment of Chemicals* (I. A. Hill, F. Heimbach, P. Leeuwangh, and P. Matthiesen, Eds.), pp. 379–398. Lewis Publishers, Boca Raton, FL.

Scorgie, H. R. A. (1980). Ecological effects of the aquatic herbicide cyanatryn on a drainage channel. *J. Appl. Ecol.* **17,** 207–225.

Snel, J. F. H., Vos, J. H., Gylstra, R., and Brock, T. C. M. Effects of the herbicide linuron on photosynthetic efficiency of freshwater macrophytes and algae. [Submitted]

Stephenson, R. R., and Kane, D. F. (1984). Persistence and effects of chemicals in small enclosures in ponds. *Arch. Environ. Contam. Toxicol.* **13,** 313–326.

Ter Braak, C. J. F. (1995). Ordination. In *Data Analysis in Community and Landscape Ecology* (R. G. H. Jongman, C. J. F Ter Braak, and O. F. R. Van Tongeren, Eds.), pp. 91–173. Cambridge University Press, Cambridge, UK.

Ter Braak, C. J. F. (1988). *CANOCO—A FORTRAN program for canonical community ordination by* [*partial*] [*detrended*] [*canonical*] *correspondence analysis, principal component analysis and redundancy analysis* (*version 2.1*). Technical Report, No. LWA-88-02. DLO-Agricultural Mathematics Group, Wageningen, The Netherlands.

Ter Braak, C.J.F. (1990). *Update Notes: CANOCO version 3.10.* Technical Report. DLO-Agricultural Mathematics Group, Wageningen, The Netherlands.

Van den Brink, P. J., Van Donk, E., Gylstra, R., Crum, S. J. H, and Brock, T. C. M. (1995). Effects of chronic low concentrations of the pesticides chlorpyrifos and atrazine in indoor freshwater microcosms. *Chemosphere* **31,** 3181–3200.

Van den Brink, P. J., Van Wijngaarden, R. P. A., Lucassen, W. G. H., Brock, T. C. M., and Leeuwangh, P. (1996). Effects of the insecticide Dursban®4E (a.i. chlorpyrifos) in outdoor experimental ditches. II. Invertebrate community Responses. *Environ. Toxicol. Chem.* **15,** 1143–1153.

Van Donk, E., Prins, H., Voogd, H. M., Crum, S. J. H., and Brock, T. C. M. (1995). Effects of nutrient loading and insecticide application on the ecology of *Elodea*—dominated freshwater microcosms. I. Responses of plankton and zooplanktivorous insects. *Arch. Hydrobiol.* **133,** 417–439.

Van Leeuwen, K., Emans, H. J., Van de Plassche, E., and Canton, H. (1994). The role of field tests in hazard assessment. In *Freshwater Field Tests for Hazard Assessment of Chemicals* (I. A. Hill, F. Heimbach, P. Leeuwangh, and P. Matthiesen, Eds.), pp. 425–438. Lewis Publishers, Boca Raton, FL.

Van Wijngaarden, R. P. A., Van den Brink, P. J., Oude Voshaar, J. H., and Leeuwangh, P. (1995). Ordination techniques for analysing response of biological communities to toxic stress in experimental ecosystems. *Ecotoxicology* **4,** 61–77.

Van Wijngaarden, R. P. A., Van den Brink, P. J., Oude Voshaar, J. H., and Leeuwangh, P. (1996). Effects of the insecticide Dursban®4E (a.i. chlorpyrifos) in outdoor experimental ditches. I. comparison of short-term toxicity between laboratory and field. *Environ. Toxicol. Chem.* **15,** 1133–1142.

Williams, D. A. (1972). The comparison of several dose levels with a zero dose control. *Biometrics* **28,** 519–531.