3.3. Design and procedure

Vessels :	100 ml Erlenmeyer flasks, cotton stoppers, on Lab-shaker, 50 ml test solution per flask
Water :	Composition according to the guideline.
Temperature :	22 ± 1°C
Lighting :	Continuous illumination, cold white fluorescent light, 115 $\mu E/m^2$ sec ± 20 % (approx. 8000 lux)
Duration :	72 hours

3.4. Stock solution

100.3 mg test substance were mixed and made up to 1000 ml with water and homogenized by ultrasonification.

3.5. Test concentrations

Nominal : 4.4, 9.6, 21, 46 and 100 mg test substance/l

Controls :
Blank : water

Replicates : Each test concentration was tested in 3 replicates, the blank control in 6.

Remarks : Calculated amounts of the stock solution to produce the desired test concentrations were given into the water and were homogeneously distributed. The algae were then transfered into the flasks.

Sampling
for analysis : Composite samples of each test concentration were drawn by mixing identical volumes of the test solutions taken from of the test vessels. They were taken immediately before exposure and after 72 hours exposure and kept at - 18°C to - 22°C until analysis.

3.6. ~~Measurements~~

Cell densities were measured at 24, 48 and 72 hours exposure on a "TOA" cell counter. For the results see tables 1 to 4 and figure 1.
Temperature was continuously measured and maintained at 22 ± 1°C.
pH was measured at 0h and 72h exposure; for results see table 5.
Analytical determination of test substance concentrations see appendix.

3.7. Calculations/Statistical Analysis

The EbC-50 values were calculated according to the maximun likelihood method, logit model (Mc Cullagh, P., Nelder, J.A., 1983: Generalized linear models, Chapman & Hall, London)

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4. Results

4.1. Values calculated

The test substance was homogeneously distributed in the test vessels at all test times and test concentrations.
The measured concentrations were within the range 100 ± 20% of the nominal concentrations (see analytical results page 17) therefore the following values were based on nominal concenrations.

a) Inhibition, areas under growth curves

EbC 50 (0-72 h) : 36 mg/l
slope :
95 % confidence limit : 28-44 mg/l

NOEbC (0-72 h) (5 % level): 9.6 mg/l

b) Inhibition, growth rates

ErC 50 (0-72 h) : 103 mg/l
slope : 1.259
95 % confidence limit : 93-117 mg/l

NOErC (0-72 h) (5 % level) : 9.6 mg/l

4.2. Values observed

none

4.3. Conclusion

Based on the EbC 50 (0-72 h) the ecotoxicological classification is according to Directive 67/548/EEC, i.e. Directive 92/32/EEC:

harmful to algae

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5. Tables and Figures

Initial cell density: 10600 cells/ml
Multiplication factor N72h / N 0h: 59

Table 1: Cell densities after 24h exposure

Conc. nominal mg/l	Dilut. Factor 1:	Cell Densities/sample cells/ml*10000 1	2	3	4	5	6	Mean Dens. N24h cells/ml*10000
Blank	10	2.6	2.4	2.2	2.2	2.2	2.1	2.3
4.4	10	2.3	2.6	2.5				2.5
9.6	10	2.4	2.7	2.6				2.5
21	10	2.4	2.3	2.7				2.5
46	10	2.8	2.9	2.6				2.7
100	10	2.6	2.5	2.5				2.5

Table 2: Cell densities after 48h exposure

Conc. nominal mg/l	Dilut. Factor 1:	Cell Densities/sample cells/ml*10000 1	2	3	4	5	6	Mean Dens. N48h cells/ml*10000
Blank	10	10.8	10.3	12.6	10.5	13.6	14.5	12.0
4.4	10	7.2	7.1	7.7				7.3
9.6	10	9.9	9.3	9.7				9.6
21	10	13.1	10.0	13.9				12.3
46	10	4.4	4.1	4.1				4.2
100	10	3.8	4.0	4.1				4.0

Table 3: Cell densities after 72h exposure

Conc. nominal mg/l	Dilut. Factor 1:	Cell Densities/sample cells/ml*10000 1	2	3	4	5	6	Mean Dens. N72h cells/ml*10000
Blank	100	72.5	73.5	54.5	53.5	51.0	68.5	62.3
4.4	100	56.5	64.5	69.0				63.3
9.6	100	52.0	59.0	68.0				59.7
21	100	37.0	34.5	39.5				37.0
46	100	22.0	21.5	20.0				21.2
100	100	8.5	9.0	9.0				8.8

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Table 4a: Inhibition

Conc. nominal mg/l	Areas under Growth Curves (A) *10000 1	2	3	4	5	6	Mean A	Inhibition * IA 0-72h (%)
Blank	1127	1123	944	882	926	1154	1026	0.0
4.4	842	943	1008				931	9.3
9.6	853	931	1045				943	8.1
21	751	646	809				735	28.4
46	371	361	336				356	65.3
100	191	199	202				197	80.8

Table 4b: Inhibition

Conc. nominal mg/l	Growth Rates (μ) *0.001 1	2	3	4	5	6	Mean μ	Inhibition * Iμ 0-72h (%)
Blank	59	59	55	54	54	58	56	0.0
4.4	55	57	58				57	0.0
9.6	54	56	58				56	0.9
21	49	48	50				49	12.6
46	42	42	41				42	26.3
100	29	30	30				29	47.8

* promotion of algal growth in regard to control is indicated as 0

Table 5: pH-values

Conc. nominal mg/l	pH 0 h	72 h
Blank	7.7	8.0
4.4	7.8	8.0
9.6	7.8	8.0
21	7.8	8.2
46	7.8	8.2
100	7.8	8.0

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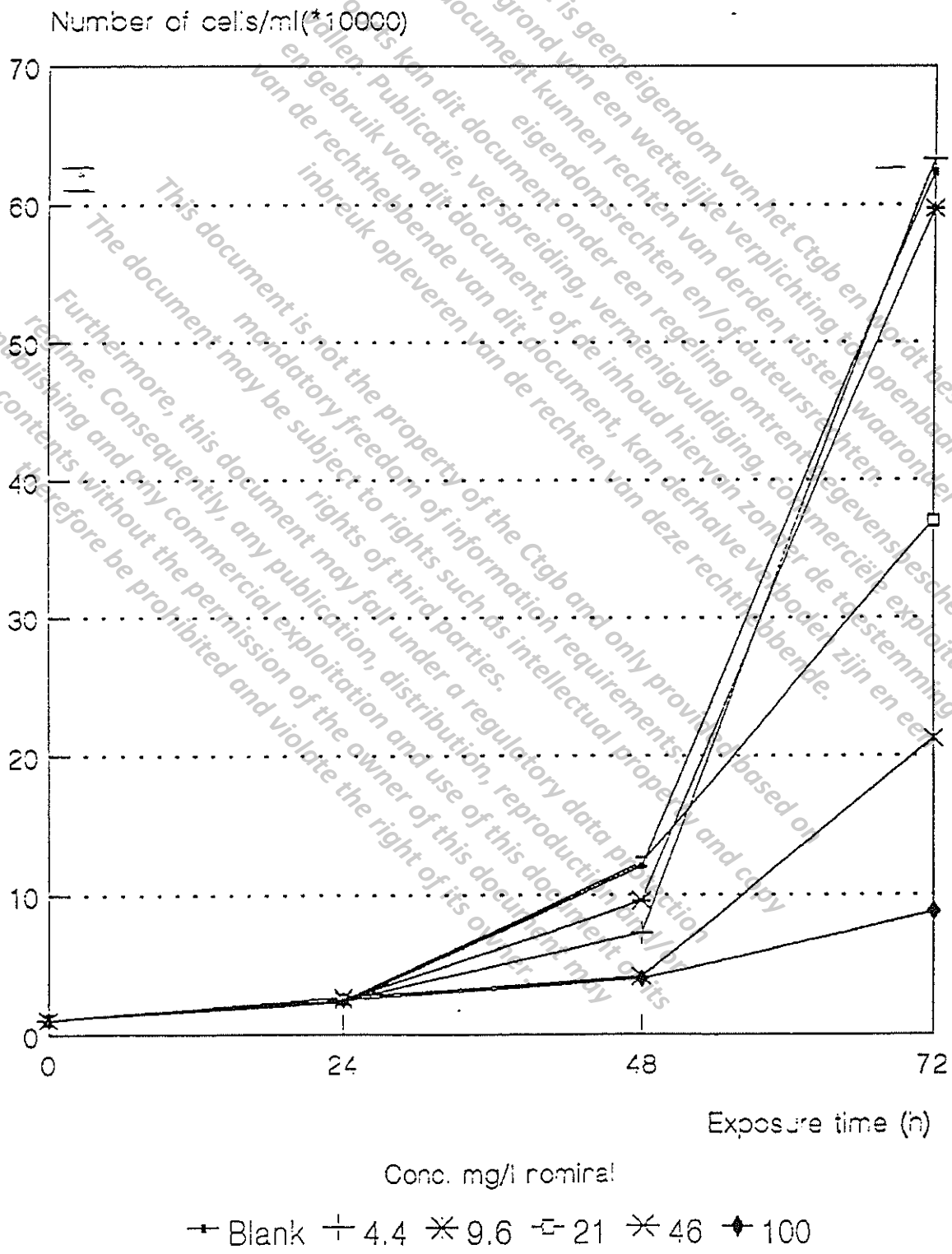
Figure 1: Growth curves



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Appendix

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ANALYSIS REPORT ON TEST NO. 933720 (PROJECT NO. OF SPONSOR: 933720
CGA 329351 WATER

DETERMINATION OF CGA 329351 IN WATER SPECIMENS FROM GROWTH INHIBITION TEST TO GREEN ALGAE

1. DESCRIPTION OF SPECIMENS
Refer to protocol of project.
Arrival of specimens: 30 May 1994.
Storage: at -20°C until analysis.
Analysis: 29 Sep 1994.

2. ANALYTICAL METHOD
General Analytical Method for "Test Substances Used for Ecotoxicity Studies", Residue Analysis, 8 Feb. 1988.
Calculations according to General Calculation Method REM 119.04.

Abstract of the method:
HPLC with UV detection: the injected specimen is preconcentrated and precleaned on a short column (C_{18}) and then transferred onto the analytical HPLC column (C_{18}). The substance is eluted with water-acetonitrile (65 vol. + 35 vol.) and detected at 240 nm.

Details of the method:
The HPLC system is equipped with a short column (1 cm length, 4 mm i.d., packed with Nucleosil 100 C_{18} 5 µm), a switching valve and an analytical column (12 cm length, 4 mm i.d., packed with Nucleosil 100 C_{18} 5 µm). 1 mL of the water specimen (appropriately diluted with water if necessary) is injected and transferred onto the short column, where it is preconcentrated and precleaned by washing with water. By means of the switching valve and water-acetonitrile (65 vol. + 35 vol.) as the mobile phase the substance is eluted from the short column and transferred onto the analytical column, from where it is eluted by the mobile phase and detected with an UV detector at 240 nm. CGA 329351 is used as the reference substance.
Quantitation: by alternate injections of water specimens and of reference substance solutions. Interpolation by method of weighted least squares of peak heights, regression of 1st order. From the measured contents of CGA 329351 the corresponding values of the test product were calculated (the product contains 97.3% CGA 329351).
The procedure was checked with recovery experiments at two spike levels. 4.5 ml of the control specimen was spiked with 0.5 ml of an appropriate standard solution of CGA 329351 in water.

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Test substance : CGA 329351

Appendix (continued)

3. RESULTS

specimen description		nominal conc. CGA 329351 techn. [mg/L]	conc. found CGA 329351 [mg/L]	conc. found (corr.) CGA 329351 techn. [mg/L]	conc. found (corr.) relative to conc. nominal [%]
24 May 94	0 h	4.4	4.13	4.10	93
24 May 94	0 h	9.6	8.91	8.85	92
24 May 94	0 h	21	20.0	19.9	95
24 May 94	0 h	46	43.6	43.3	94
24 May 94	0 h	100	97.0	96.3	96
27 May 94	72 h	4.4	3.94	3.91	89
27 May 94	72 h	9.6	8.38	8.32	87
27 May 94	72 h	21	18.3	18.2	87
27 May 94	72 h	46	40.5	40.2	87
27 May 94	72 h	100	88.0	87.4	87
24 May 94	0 h	control	<1.00	<0.99	–
27 May 94	72 h	control	<1.00	<0.99	–

Remarks:
- conc. found (corr.): these results are corrected for an average recovery of 103.5 %.

Recoveries:
Spike level 2.0 mg/L CGA 329351 (2.1 mg/L CGA 329351 techn.): 103%
Spike level 8.0 mg/L CGA 329351 (8.2 mg/L CGA 329351 techn.): 104 %

Analyst: 5.1.2.e Woo

25 NOV 1994
date

5.1.2.e Woo
(principal investigator for analytics)

Distribution: Dr. 5.1.2.e Woo (study director)

Original report and raw data in archives of Residue Analysis, PP 2.53.