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Influence of the growth pattern on the EC_{50} of Cell Number, Biomass Integral and Growth Rate in the Algae Growth Inhibition Test

Volume I

Anonymized Version

by

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CONTENT

SUMMARY 5

ZUSAMMENFASSUNG 6

1. GENERAL INTRODUCTION 7

2. RESPONSE VARIABLES IN THE AGIT AND THEIR PROPERTIES - REVIEW OF THE STATE OF THE ART. 8

2.1 Background 8

2.2 Cell number 10

2.3 Biomass integral 10

2.4 Growth rate μ 10

2.5 .Threshold and effect concentrations as toxicity parameters 11
2.5.1 Threshold concentration (NOEC) 11
2.5.2 Effective concentrations, ECx 12

2.6 The difference between the EC_x for the biomass parameter and growth rate 13

2.7 Discussion 14

3. RATIONALE FOR THE PRESENT STUDY 15

4. ANALYSIS OF SUBMITTED TEST REPORTS 16

4.1 Introduction 16

4.1 Methods 16
4.1.1 Calculation of response-variables 16
4.1.2 Statistical properties of the response variables 17
4.1.3 Determination of the NOEC (threshold concentration) 18
4.1.4 Computation of effective concentrations ECx 19
4.1.5 Software 19

4.2 Detailed description of the growth course 19
4.2.1 Substance 1 20
4.2.2 Substance 2 20
4.2.3 Substance 3, 4 20
4.2.4 Substance 5 21
4.2.5 Substance 6 21
4.2.6 Substance 7 21
4.2.7 Substance 8 22
4.2.8 Substance 9 22
4.2.9 Substance 10 22
4.2.10 Substance 11 23
4.2.11 Substance 12 23
4.2.12 Substance 13 23

4.2.13 Substance 14 24
4.2.14 Substance 15 24
4.2.15 Substance 16 24
4.2.16 Substance 17 25
4.2.17 Substance 18 25
4.2.18 Substance 19 25
4.2.19 Substance 20 26
4.2.20 Substance 21 26
4.2.21 Substance 22 26
4.2.22 Substance 23 27
4.2.23 Substance 24 27
4.2.24 Substance 25 27
4.2.25 Substance 26 28
4.2.26 Substance 27 28
4.2.27 Substance 28 28
4.2.28 Substance 30 29
4.2.29 Substance 31 29
4.2.30 Substance 32 29
4.2.31 Substance 33 30
4.2.32 Substance 34 30
4.2.33 Substance 35 30
4.2.34 Substance 29 31
4.2.35 Substance 36 31
4.2.36 Substance 37 31
4.2.37 Substance 38 32

4.3 Interpretation of the growth pattern by characteristic scenarios 32
4.3.1 Introduction 32
4.3.2 Basic growth pattern: the exponential growth 32
4.3.3 Growth modification due to experimental errors and shortcomings 33
4.3.4 Growth modification due to test-substance effects 36

4.4 Results on basic test parameters 39

4.5 Comparison of toxicity parameters 40

4.6 The ratio between the ECx in various response variables 41
4.6.1 The ratio E_rC_{50}/E_bC_{50} 41
4.6.2 Reasons for extreme ratios of E_rC_{50}/E_bC_{50} 42
4.6.3 Comparison of EC_{50} ratios of various response variables 48
4.6.4 Comparison EC_{10}, EC_{20} and EC_{50} ratios of various response variables 49

4.7 Concluding remarks about the results with submitted test data 54

5. EFFECTS OF GROWTH COURSE ON THE RESPONSE VARIABLES - THEORETICAL EXAMPLE SCENARIO ANALYSIS 55

5.1 Introduction 55

5.2. The effects of experimental time, slope of the growth curve and slope of the dose/response curve on the response variables 56
5.2.1 The effects of experimental time on the response variable values 56
5.2.2 The effects of slope of the dose/response curve on the response variable values 57
5.2.3 The effects of the slope of the growth curves on the response variable values 59

5.3 The effects of dissimilar distribution of data points within the dose/response range on the response variables 61
5.3.1 Dissimilar distribution at zero variability 61
5.3.2 Dissimilar distribution and the effects of variability 63

5.4 Influence of the growth pattern on the toxicity parameters and their ratios 70
5.4.1 Introduction 70
5.4.2 Effects of growth modification, caused by experimental shortcomings, on the toxicity parameters 70
5.4.3 Effects of growth modification due to test-substance effects 73
5.4.4 Conclusions from the simulations 75

6. FINAL CONCLUSIONS 76

6.1 Test design and experimental conditions 76

6.2 Growth pattern 76

6.3 Statistics 77

6.4 Toxicity parameters 77

6.4 Response variables 77

7. REFERENCES 79

ANNEX A 81

A1 Results on basic test parameters 81
A1.1 Statistical distribution and variance homogeneity of response variables - prerequisites for parametric test methods 81
A1.2 Prerequisites for dose/response curve modelling to derive an ECx 83
A1.3 The behaviour of test parameters used or discussed as validity criterion 87

A2 Comparison of toxicity parameters 88
A2.1 Submitted results vs. those from the present study 88
A2.2 Statistical test, data transformation and the NOEC 88
A2.3 Comparison of EC_{10}, EC_{20} and EC_{50} with the NOEC 93

A3 Toxicity parameter values obtained with the scenarios of dissimilar distribution of the data points 106

A4 Toxicity parameter values obtained with the scenarios identified as basic for the submitted tests 109

Summary

In the present study, an analysis of the algae growth inhibition test was performed, which included the theoretical background of response variable selection, the statistical properties of possible response variables, the statistical analysis of test results, the reasons for differences in toxicity parameters, and the interpretation of the test results. This was done by means of a literature survey, by an analysis of a selection of 38 test reports, provided by the German Federal Environmental Agency, and by simulated tests, allowing the investigation of several test scenarios systematically.

In a substantial number of the submitted tests, there was strong evidence for shortcomings in the preparation and conduct of the tests, ranging from inappropriate pre-culture of algae, leading to lag-phases, to bad selection of concentration range and spacing. Except for a temporary inconstancy, e.g. in temperature or light conditions, all of the discussed disturbances of growth impeded the correct assessment of the toxicity. Especially, if the concentration/inhibition data points were inappropriately located on the dose/response curve, mathematics was not able to find the appropriate dose/response function, thus leading to false estimates of toxicity.

The basic growth pattern was exponential in many cases – at least for some period of time. However, toxicants exhibiting a timely changing toxicity change the strict exponential pattern. Other deviations were caused by experimental shortcomings. Nonetheless, no reasons were found to reject the hypothesis of the exponential growth as the basic growth form.

For use with parametric statistical testing, as has to be conducted for the determination of a NOEC, the statistical properties of two growth rate variants (the average growth rate and the section by section growth rate), the cell count (also the log-transform) and the biomass integral were examined. The growth rate variants and log(Cell count) proved to be more appropriate than the cell count and biomass integral (small variance, variance homogeneity, normal distribution). For the determination of a NOEC, Williams test procedure was superior over Dunnett's test or the non-parametric Bonferroni-U test.

Also for dose/response modelling, the low variance in the average growth rate appears beneficial in finding the appropriate function and in computation of confidence limits of the ECx.

Many of the results from the submitted reports and the present study differed, indicating that the statistical methods for determination of the toxicity parameters need standardisation. If the NOEC shall be replaced by some ECx in the future, the EC_{10} appears more appropriate than another ECx, where $x > 10$.

Extreme EC_{50}-ratios between the growth rate and the biomass parameter were clearly due to an inappropriate choice of the concentration range. It was found that the same concentration range is not optimal for all of the response variables. Therefore, it is desirable to select only one of the variables and optimise the concentration range for this variable.

The criterions for selection the appropriate response variable can be different: relevance for population dynamics, sensitivity, statistical confidence. In the scientific literature, the growth rate is favoured as a population parameter of prime importance and this is seen as the reason to select the growth rate as the alone response variable in a test.

Overall, also according to the present study, sound reasons - ranging from the ecological relevance, correct toxicity assessment to statistical power – are in favour of the preference of the average growth rate as the basic response variable for algal growth and for the consistent assessment of toxicity.

Zusammenfassung

In der vorliegenden Studie wurden Algenwachstumshemmtests analysiert, um sowohl die theoretischen Hintergründe für die Auswahl von Messvariablen des Algenwachstums, als auch die statistische Methodik, Ursachen für Unterschiede in den Toxizitätsparametern und die Interpretation von Ergebnissen zu beleuchten. Hierzu wurde eine Literaturstudie durchgeführt, Resultate aus einer Auswahl von 38 Testberichten analysiert, die das Umweltbundesamt zur Verfügung stellte, und Algenwachstumshemmtests simuliert, um bestimmte Test-Szenarios systematisch zu untersuchen.

In einer nennenswerten Anzahl von Tests aus der Praxis dürften Mängel in der Testvorbereitung und- durchführung, z.B. fehlerhafte Vorkultur der Algen, was zu lag-Phasen im Wachstum führte, ungeeignete Auswahl der Testkonzentrationen und der Konzentrationsstufung, für Fehler in der Toxizitätsschätzung verantwortlich sein. Insbesondere in solchen Fällen, in welchen die Messpunkte für die Hemmung des Biomasseparameters und der Wachstumsrate eine ungleichmäßige Verteilung im Dosis/Wirkungsbereich zeigten, war die mathematische Auswertung nicht in der Lage, die wahre Dosis/Wirkungskurve zu ermitteln.

Das grundlegende Wachstumsmuster war in vielen Fällen exponentiell – mindestens für eine gewisse Zeitperiode. Jedoch rufen Substanzen, deren Toxizität sich mit zunehmender Versuchszeit ändert, auch Abweichungen der exponentiellen Form der Wachstumskurve hervor - ebenso auch experimentelle Mängel. Dennoch besteht keine Veranlassung, von der Hypothese eines exponentiellen Wachstums als Grundmuster Abstand zu nehmen.

Zwei Varianten der Wachstumsrate (die mittlere Wachstumsrate und die abschnittsweise Wachstumsrate), die Zellzahl (auch in log-Transformation) und das Biomasseintegral wurden im Hinblick auf ihre statistischen Eigenschaften geprüft, da diese für die Bestimmung einer NOEC mittels statistischem Test wichtig sind. Am geeignetsten erwiesen sich die zwei Wachstumsraten-Varianten und die log-transformierte Zellzahl (geringe Varianz, Varianzhomogenität, Normalverteilung). Für die Bestimmung der NOEC empfahl sich der Williams-Test vor dem Dunnett-Test und dem nicht parametrischen Bonferroni-U-Test.

Auch für die Modellierung der Dosis/Wirkungsfunktion erscheint die geringe Varianz der mittleren Wachstumsrate für die Auffindung einer geeigneten Funktion und die Berechnung von Vertrauensintervallen der ECx vorteilhaft zu sein.

Die Ergebnisse in den eingereichten Testberichten wichen vielfach von denen aus der vorliegenden Studie ab. Hieraus ergibt sich eine Notwendigkeit, die statistischen Methoden zu Bestimmung der Toxizitätsparameter zu standardisieren. Sollte die NOEC künftig durch irgendeine ECx ersetzt werden, so erscheint die EC_{10} eher geeignet als eine höhere ECx (mit $x > 10$).

Extreme EC_{50}-Relationen zwischen Wachstumsrate und Biomasseparameter waren eindeutig auf fehlerhafte Konzentrationswahl zurückzuführen. Dieselbe Konzentrationsreihe erwies sich als nicht optimal für alle Messvariablen. Daraus ergibt sich, dass nur eine Messvariable bevorzugt werden sollte, für die dann die Konzentrationsauswahl optimiert werden kann.

Die Kriterien für die Auswahl einer geeigneten Messvariablen können unterschiedlich sein: sie umfassen die populationsbiologische Relevanz, Sensitivität und statistische Vertrauenswürdigkeit. In der wissenschaftlichen Literatur wird der Wachstumsrate wegen ihrer überragenden populationsbiologischen Bedeutung der Vorrang eingeräumt.

Zusammenfassend ergaben sich auch aus der vorliegenden Studie gute Gründe – ökologische Relevanz, korrekte Schätzung der Toxizität und statistische Power – der mittleren Wachstumsrate den Vorzug als der grundlegenden Messvariablen zu geben, weil hauptsächlich sie eine zuverlässige Schätzung der Toxizität erlaubte.

1. General Introduction

The Algae Growth Inhibition Test (AGIT) belongs to the base set of ecotoxicological tests, conducted in order to assess the hazard potential of pesticides and chemicals for the aquatic environment[1]. Table 1 presents a list of some of the currently used guidelines[2]. However, since the development of these guidelines, prescribing the conduct, data evaluation, and statistical analysis of the AGIT as well as the reporting requirements, there are still debates about the selection of culture media (ISO 1997), the test duration, applicability for coloured substances, and selection of the appropriate response variables (growth rate vs. biomass integral or cell number). These variables represent different measures of algal growth, being the toxicant-affected target (endpoint). The discussions on response variables were stimulated by both theoretical considerations and sometimes unexpected results from AGITs, submitted for the registration of pesticides. Among these results are extreme differences in the ratio E_bC_{50} / E_rC_{50}. The EC_x (x = 10, 50) are among the most used toxicity parameters for the response variables in an algae test.

Table 1: Overview over a selection of guidelines/standards, currently used for the conduct of the AGIT; Ss: *Scenedesmus subspicatus*; Sc: *Selenastrum capricornutum*[3]; Cv; *Chlorella vulgaris*; Skc: *Skeletonema costatum*; Af: *Anabaena flos-aquae*; A: biomass integral, µ: mean growth rate; CC: cell count / biomass.

Guideline / Standard	Test species	Test Duration [h]	Response variable(s)	Toxicity Parameter
DIN 38412 L9 (DIN 1993)	Ss, Sc	72	A, µ	E_bC_{10}, E_bC_{50}, E_rC_{10}, E_rC_{50}
OECD 201 (1984)	Ss, Sc, Cv	72	A, µ	NOE_bC, E_bC_{50}, NOE_rC, E_rC_{50}
EPA-FIFRA	Sc, Skc, Af	120	CC	NOE_cC, E_cC_{25}, E_cC_{50}
ASTM – E 1218-90	Sc, other	96	CC or A and/or µ	E_cC_{50} or E_bC_{50}, and/or E_rC_{50}
ISO 8692 (1989)	Sc, Ss	72	A, µ	NOE_bC, E_bC_{50}, NOE_rC, E_rC_{50}
EU (1992)	Sc, Ss	72	A, µ	NOE_bC, E_bC_{50}, NOE_rC, E_rC_{50}

Generally the AGIT comes as one of those biotests, the conduct of which is hardly problematic, provided the prescriptions of the test guidelines can be followed and the test conditions appropriately controlled. In practice, however, growth curves from algae cultures often show irregularities and the values obtained for the response variables appear hardly logical. Besides errors in the experimental conduct and statistical evaluation, properties of the test substance and the culture medium, as well as the performance of the algae cells can affect growth at different time periods during the test, thus leading to growth curves deviating from the expected monotonously exponential ones. Hence, with respect to the interpretation of test results there is urgent need to distinguish between the various factors affecting algal growth – in particular between substance properties and experimental or computational shortcomings or errors. According to most of the current test guidelines, a biomass parameter (cell number, biomass integral) and the growth rate has to be determined, the substance-caused inhibition of which has to be shown by means of some toxicity parameter. Among these are the so-called effect concentrations, EC_x (e.g., x = 10, 50), or a threshold concentration (e.g., NOEC). It is common practice, to use the preferred toxicity parameter from that response variable which exhibits the lowest value (often called "most sensitive endpoint")[4], for regulatory purposes. Therefore, in order to generate scientifically sound and reproducible risk assessments, there exists urgent demand to investigate the possible reasons for the various sorts of results ob-

[1] Algae Growth Inhibition Tests for effluent testing are not considered in this study.

[2] To be brief, for the following, „guideline" is used as comprehensive term for "guideline", "standard", "technical paper", etc

[3] The correct new name is *Pseudokirchneriella subcapitata*, but the older name will be used throughoutly, because is used in the current literature and guidelines

[4] I prefer to use the term "sensitive endpoint" for the biological process, the algae growth.

served with practical testing. Are large deviations in the response-variable values from the same test, as observed frequently and demonstrated again by examples in the present study, due to unacceptable shortcomings/mistakes during testing and statistical evaluation? Have these deviations to be attributed to acceptable causes, e.g. nature of the response variables, properties of the test substance and algae species? Which response variable can be used as consistent and confident estimate of the toxic inhibition of algal growth?

In the present study, the current literature on this topic will be reviewed at first and a selection of 38 test reports from AGITs, provided by the German Federal Environmental Agency, will be analysed. In addition, also simulated AGITs are used, by which several test scenarios were investigated systematically. This approach intends to aid the interpretation of the observed results and allows comparing "true" effects – being set for the simulations – with those observed by the evaluation method for all of the response variables.

2. Response variables in the AGIT and their properties - Review of the state of the art.

2.1 Background

The discussion about the selection of the appropriate response variable in algal growth inhibition tests has been going on controversially since the early eighties (e.g., Nusch 1982, 1983, Nyholm 1982, 1985). The response variables include „average growth rate" (μ) and "biomass" (≈ cell count) or „biomass integral" (A, area under the growth curve) being favoured by their respective advocates. Because there has been no consensus about the preferred variable, current guidelines prescribe that a biomass parameter (in most cases the biomass integral) and the growth rate should be calculated (Table 1). So, the selection of the most appropriate variable is left to the regulator, who normally chooses the lower effective or threshold concentration (EC_{50}, NOEC) for the notification or the risk assessment. Before the pros and cons for these parameters will be thoroughly discussed, a short introduction about these variables and their computation will be presented.

The base of algal test evaluation is the population growth curve, obtained for a defined test duration (most frequently 72 hours). At the start of the test, an algae inoculum is transferred into the culture medium, providing a defined nutrient content. For the treatments, increasing amounts of a test substance are added. The unicellular coccoid green algae *Scenedesmus subspicatus* and *Selenastrum capricornutum* (new name: *Pseudokirchneriella subcapitata*) are among the most often used test species. At the start of a test, the cell density should be 10000 cells/mL and the inoculum should be taken from an exponentially growing algal pre-culture, which has been set up 72 hours before the start of the test under the same conditions like in the test.

For each tested concentration normally three replicates per treatment and six replicates for the control are incubated. During the test the algae population grows exponentially, in case there are no limiting conditions (e.g., nutrients, light, and toxicant). At least in the control, these conditions should be prevailing. The exponential growth is described by the Equation 1.

$$N_t = N_0 \, e^{\mu t} \qquad (1)$$

with

N_t	cell number at time t
μ	growth rate of the population
N_0	cell number at time t_0
t	time

N_t, the mean cell densities, are measured after 24, 48 and 72 hours and – plotted over time – form the growth curve of a control or treated population. Figure 1 gives an example of growth curves, obtained with a test with potassium dichromate ($K_2Cr_2O_7$) in the laboratory of the authors.

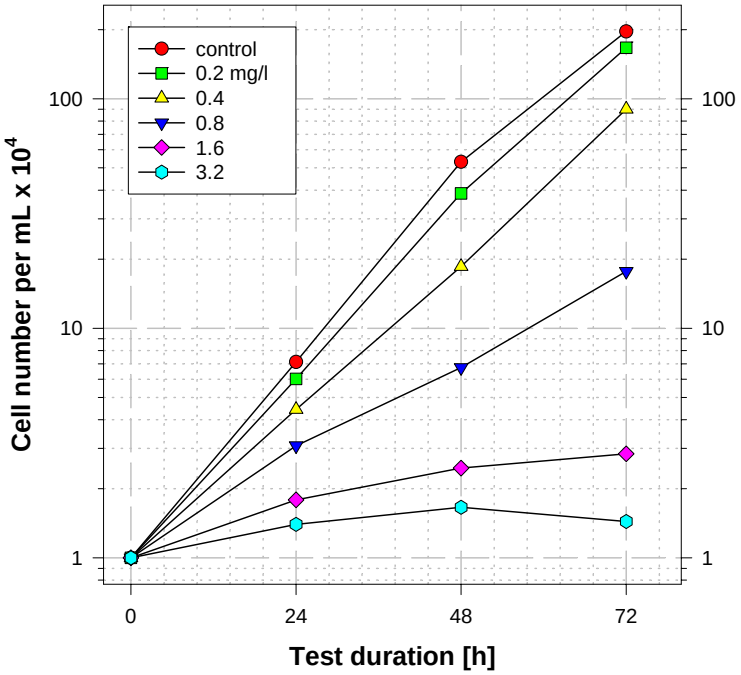


Figure 1: Growth curves of the control and five tested concentrations of potassium dichromate.

Due to the reaction of a test substance with target bio-molecules of the inner cell or cell membrane, the algal metabolism can be affected, resulting either in cell death (lethal effect, algaecidal effect) or disturbance of metabolic reactions, leading to reduced cell growth and division rate (sublethal effect). Both the (partial) cell mortality and sublethal effects reduce the population growth rate, μ, thus leading to the production of fewer cells (biomass) per time unit. Therefore, the cell number at different times during the test is the primary measure of the growth process and its inhibition. In addition, also derived measures are used: the growth rate and the biomass integral. Up to now there is no consensus whether the cell number (US-EPA 1982), the biomass integral (the area under the growth curve, Nusch 1982, 1983), or the evaluation using the average growth rate (Nyholm 1985, 1990, 1994) shall be considered as the definitive response variable of the endpoint “algal growth”. Subsequently, the discussion about the three variables will be summarised.

2.2 Cell number

The cell number per volume at the end of the test, C_t, is a surrogate measure of the ultimate algal biomass or yield, which normally is expressed in terms of weight or volume. Nyholm (1985) points out that this variable, although being influenced by toxicants, does not provide a good response variable for toxicity tests: ”The reasons include: (1) even considerable reductions in growth rate may not result in changes or may be associated with only minor changes in final yield, because the toxicant-affected may gradually ‘catch up’ with the controls when nutrients become limiting; (2) in the course of the test, toxicity can be lost due to various mechanisms and thus cause little or no effect on the final yield.”

2.3 Biomass integral

The area under the growth curve as a biomass-related variable was proposed by Nusch (1982, 1983; area comparison method). This parameter is a theoretical construct, and, although a meaning in population dynamics is lacking, its advantage is seen in the fact that there is – as with cell number - no assumption about the mode of growth, but in contrast to the cell number, determined at the end of the test, the complete course of the growth curve is exactly represented – including all irregularities. The biomass integral is computed using a trapezoidal integration (Equation 2). The non-transformed values of the cell numbers (at 24, 48 and 72 hours) are connected with straight lines, and the single areas are computed and summed up.

$$A = \frac{N_1 - N_0}{2} t_1 + \frac{N_1 + N_2 - 2N_0}{2}(t_2 - t_1) + \ldots + \frac{N_{n-1} + N_n - 2N_0}{2}(t_n - t_{n-1}) \qquad (2)$$

with

$N_{0,\,1,\,2,\,n}$: cell number at time t_0 (start of the test), t_1, t_2, t_n

$t_{1,\,2,\,n}$: time of the 1st, 2nd, nth measurement after the start of the test

This method integrates all measurements of the algal cell density during the test duration. Therefore, it is suitable to detect irregularities during the growth process of the population.

2.4 Growth rate μ

The average growth rate (μ; sometimes termed μ_{av}) describes the average slope of the growth curve during the test duration. The computation follows Equation 3.

$$\mu = \frac{\ln N_t - \ln N_0}{t} \qquad (3)$$

with:

N_0: cell number/mL at time t_0 (start of the test)
N_t: cell number/mL at time t
t: test duration in days

The average growth rate is a logarithmic quantity (unit: d^{-1}). Small differences in the average growth rate cause relatively large differences in the cell number. The average growth rate is

determined from two measurement points at the start and the end of the test. However, the start density, N_0, should be derived from the dilution of the algae stock solution rather than from density measurements in the test vessels at time t_0, since at low algal densities sampling and measurement errors are substantially high, thus influencing the exact computation of μ (Nyholm 1985).

2.5 .Threshold and effect concentrations as toxicity parameters

2.5.1 Threshold concentration (NOEC)

For the biomass variable (cell number, biomass integral) and the growth rate, a No Observed Effect Concentration (NOEC) has to be reported according to various guidelines (Table 1). Although the NOEC as toxicity parameter will be phased out for the future (OECD 1998), its determination shall be briefly described, since it is still used in the regulatory process by some authorities. The NOEC is a threshold concentration, which is determined by a multiple statistical test (e.g., Dunnett's test procedure; Dunnett 1955, 1964; Williams test, Williams 1971, 1972; Bonferroni-U test, Holm 1979). The test is performed using the replicate values of the biomass parameter and of the growth rate. According to EU (1992), the replicate values for the biomass integral and growth rate should be calculated for each test vessel (replicate), before treatment means and variances are computed. For multiple testing the pooled variance estimate, obtained with an one-way ANOVA, should be preferred.

The conduct of the Dunnett-test is very similar to the commonly used STUDENT t-test. However, the significance level (α-error, type-I-error) is adjusted to the whole experiment rather than the single pair-wise comparison and a correction for unequal replication has to be included. The multiple approach then allows the following statement: The concentration x and higher ones cause an effect, whereas at the same time those lower x do not have a statistically significant effect. A statement like this takes into account all of the treatments including the control. Hence, also the type-I-error must reflect this and be adjusted to the whole experiment rather than to a simple pair-wise comparison. Table 2 gives an example, how the NOEC for Substance 2 was determined by the Dunnett test. According to the results of the statistical test, a NOEC of 0.046 mg/L for 0 - 24 h, and 0.1 mg/L for 0 - 48 h or 0 - 72 h can be derived.

Table 2: Comparison of the cell densities in Substance 2-treated and control cultures at various times using Dunnett´s-Test at a significance level of 5 % (one-sided); results which were significantly different from the control are marked with an asterisk (*).

Concentration (mg/L)	Calculated t-Value 0 - 24 h	0 - 48 h	0 - 72 h
0.046	2.11	-1.90	-1.64
0.1	6.02 *	2.02	-0.04
0.21	7.46 *	5.67 *	6.86 *
0.46	8.31 *	9.05 *	12.64 *
1	11.36 *	16.41 *	19.31 *
Tabled t-Value	2.28	2.28	2.28
Degrees of Freedom	57	57	57
Total Variance	0.85941	29.122	352.71

In the present study, NOECs were determined in order to compare these with the EC_{10}, EC_{20} and EC_{50}.

2.5.2 Effective concentrations, ECx

Effective concentrations are concentrations which cause a distinct effect quantity, x, on a response variable. For example the EC_{10} or EC_{50}, both required by e.g. DIN (1993), are those concentrations, which cause 10% and 50% reduction/inhibition in the considered variable. In contrast to the NOEC, being determined by a statistical test, these values are derived from so-called data modelling, because of the fact that a given ECx hardly is one of the test concentrations. Therefore a function is fitted to all of the treatment means of the considered variable. Although a direct modelling of a function for the reduction in cell number, biomass integral, and growth rate is principally possible, the commonly used guidelines prescribe that the inhibition relative to the control of these parameters is computed first using Equation 4.

$$I = 1 - \frac{P_T}{P_C} \qquad (4)$$

with:

P_C: arithmetic mean of the parameter (C, A, μ) in the control
P_T: arithmetic mean of the parameter (C, A, μ) in a treatment

The inhibitions (often also expressed as percentage inhibitions) at different concentrations describe a specific concentration/response curve, to which in many cases a normal sigmoidal function can be fitted using probit analysis[5], based on a maximum likelihood regression analysis (Finney 1971, 1978). But also the logistic and Weibull function – sometimes also used with non-linear regression analyses - were demonstrated to give reliable fits to the data (Christensen & Nyholm, 1984, Christensen 1984, Voelund 1978). The principal differences between these models are demonstrated in Figure 2.

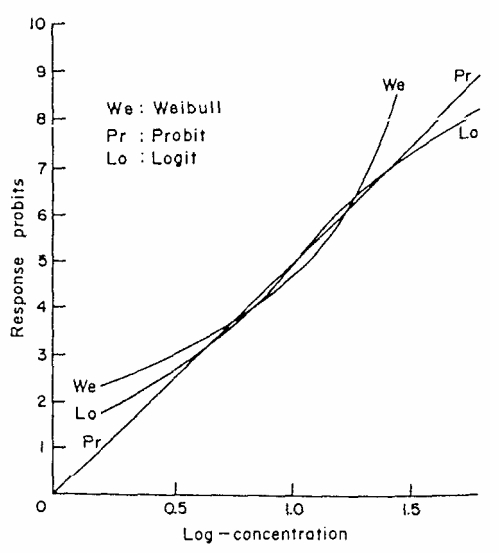


Figure 2: Schematic comparison of the Weibull, probit and logit models (from Christensen 1984)

Although these models are mainly empirical, there are suggestions to interpret the slope of the Weibull and logit model as a measure for the reacting toxicant molecules per active receptor (Christensen & Nyholm 1994). By a selection of test data, these authors showed that the EC_{50}s, calculated using the three models do not substantially differ, but the Weibull-model

[5] Please note that probit analysis was "invented" for quantal responses. For quantitative parameters, such as in algal test variables, the weighting for regression analysis and the computation of the residual variance and confidence limits have to be modified (see Christensen 1984)

leads generally to lower EC_{10} and EC_{90} values, which can also be concluded from the Weibull curve shown in Figure 2. These differences could be important, since the EC_{10} (or any EC < 25) will probably replace the NOEC in future.

2.6 The difference between the EC_x for the biomass parameter and growth rate

The EC_x-values for the biomass parameter (E_bC_x) are found normally lower than those for the average growth rate (E_rC_x; Hanstveit & Oldersma 1981, Hanstveit 1982, Dorgerloh 1997). For the example in the test with potassium dichromate, shown in Figure 1, the E_bC_{50} was 0.34 mg/L, whereas the E_rC_{50} exceeded this value by 2.5 fold (0.86 mg/L; ratio 0.4).

Nyholm (1985) analysed this problem mathematically. Under the assumption of exactly equal cell density at the start and strictly exponential growth, Equation 5 was derived for the ratio E_bC_{50}[6] / E_rC_{50}.

$$E_bC_{50} \Big/ E_rC_{50} = 10^{\left[\frac{1}{\alpha}\left(\frac{\ln 2}{\mu_m \bullet t} - 0{,}5\right)\right]} \qquad (5)$$

with:

μ_m: maximum growth rate of the control
α: slope of the dose response curve (relative growth rate vs. log concentration)
t: time

Characteristics of this function are demonstrated in Figure 3. The ratio approaches infinity as α tends to zero (very flat dose response curves), while for large α´s („threshold“ or „all or nothing types“ of effects) the ratio approaches unity. For a test duration shorter than t = 2 ln2/μ (e.g., 0.77 days for μ = 1.8 d^{-1}) the E_bC_{50} is larger than the E_rC_{50}, while for a longer test duration the E_bC_{50} is smaller than E_rC_{50}.

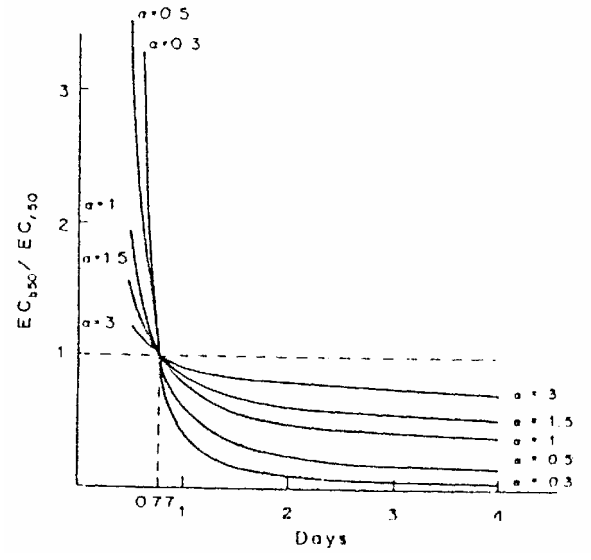


Figure 3: Plot of the ratio E_bC_{50} / E_rC_{50} as calculated from Eq. 5, assuming a specific growth rate of μ = 1.8 d^{-1}; α is the slope of the dose response curve for relative growth rate vs. log concentration (after Nyholm 1995)

According to this function, a longer test duration, high growth rate, and flat dose-response relationship are expected to evoke large differences between the E_bC_{50} and E_rC_{50}. According

[6] Note that Nyholm made these analysis for the biomass (e.g., weight, cell number) rather than for the biomass integral. However, the results for the biomass integral are principally the same.

to additional theoretical considerations of Nyholm (1985), the EC_{10} is expected to be less dependent on the endpoint selected.

2.7 Discussion

The following section summarises the discussion about the appropriate response variables as found in the published literature. Views of the authors of the present study will be given in the final chapter, after the results of the present study are presented and discussed.

For the use of the response variable “biomass” (measured as cell density) above all practical arguments are put forward, like (1) simplicity, (2) direct interpretation of the results without any assumptions necessary on the mode of growth, (3) generation of generally lower and thus more “sensitive” EC_x-values and (4) toxic effects can be detected more easily since small changes in growth rate cause larger changes in biomass.

Supporters of the response variable “growth rate” reply that EC_{50}´s derived from biomass are strongly influenced by test design parameters such as test duration, exact adjustment of cell density at the start of the test, occurrence of lag-phases and the maximum growth rate (and thus by a variety of other test specific parameters) of the algae. Therefore, these data appear to be only badly reproducible. In contrast, the toxicity data derived from growth rate are rather independent from the test conditions and thus appear more robust against such deviations (Källqvist et al. 1980, 1982). Supporters of the biomass point out that the reproducibility of the test results is sufficient for most purposes and that the EC-values should be only of same magnitude, while growth rate supporters argue that the reproducibility should be as high as possible, if this does not or only negligibly increase the costs.

The apparent advantage of lower EC_x-values for biomass is seen simply as a mathematical consequence of the exponential growth pattern and is additionally combined with a larger variance of the data (Nyholm & Källqvist 1989). Thus it is doubtful, whether a toxic effect really can be detected earlier (and statistically improved). The larger variance of the data derived from biomass could possibly be diminished by strict standardisation of test conditions, although supporters of the growth rate see a danger of „over-standardisation“.

Although the resulting biomass is a direct consequence of the toxic influence on growth, it depends on further factors like the biomass at the start of the test and the test duration. Therefore effects depending on the test design are mixed inevitably with the evaluation of the toxic effect. The independence of the growth rate and the derived EC-values from the test duration is particularly of striking importance if the test duration will be flexible (e.g. to avoid problems like pH-variation, self shading or limitation of nutrients, Nyholm 1994). In this case, the use of the average growth rate as endpoint is seen as inevitable.

Some critics refuse the growth rate, because they see strict exponential growth as a prerequisite for using the growth rate and doubt that this applies for most of the tests. However, by inoculating with algal cells from an exponentially growing pre-culture and a low cell density (10000 cells/mL) at least in the control and the samples with lower (0-50%) toxic effects exponential growth normally takes place. Furthermore also the growth in stronger inhibited samples is usually closer to exponential than to linear growth.

An advantage of using the response variable “biomass integral” is seen in that all measured cell numbers (24, 48, 72 h) are used for the evaluation (e.g., Nusch 1982, 1983). In so doing, also transient effects (lag phases or special kinetics of the test substance) are part of the evaluation, while the average growth rate is usually determined by only two measurement points at the start and the end of the test, so that temporary effects can be overlooked. However, it is doubted that a weak inhibition at 24 or 48 hours has an important influence on the

test populations, because it can be often noticed that after 72 hours such a weak effect is already compensated and thus a remaining influence on the algal population is not measurable any more (Nyholm 1982, 1985).

This was a report on the discussions found in published papers so far. A final assessment of the pros and cons of the various response variables will be given in Chapter 6, after the results of the analysis of a selection of AGITs have been presented and discussed.

3. Rationale for the present study

As can be concluded from the above literature survey, the ongoing discussions about appropriate response variables in the algal test started more than ten years ago and the reasoning was partly based on theoretical considerations. The simultaneous use of two response variables for the same endpoint is seen as the non-ideal case by regulatory authorities and industry. This is especially due to the fact that in practice unexpected high differences between the ECx of the biomass parameter and that of the growth rate are to be observed. Therefore, before any future modification of guidelines will be performed, there is urgent need to conduct an analysis of the algae growth inhibition test procedure and its results. This analysis should include:

- the theoretical background of response variable selection
- the statistical properties of possible response variables
- the statistical analysis of test results
- the reasons for differences in toxicity values
- the interpretation of the test results

With respect to the interpretation and assessment of test results, one of the most important issues is to distinguish between special behaviour and action of test substances and shortcomings or errors in the conduct of the test and statistical analysis of the test results, both strongly influencing the quantity of response variables.

By means of a selection of tests results, submitted to the German Federal Environmental Agency, and simulated AGITs using theoretical scenarios the present study intends to close gaps in knowledge about the behaviour of the various response variables and to present theoretical and practical background for future guideline development.

4. Analysis of Submitted Test Reports

4.1 Introduction

The submitted test reports, evaluated anew by the present study, are presented in Table 3 and 4, together with some information about basic characteristics of the tests. For the following, a test report will be identified by the name (or abbreviation) of the test substance examined. From the variety of test reports, submitted to the German Federal Environmental Agency, those were selected which showed extreme differences in the ratio E_rC_{50}/E_bC_{50} (Table 4), as well as those with apparently "normal" ratios ($1 < E_rC_{50}/E_bC_{50} < 10$; Table 3)[7].

The analysis of tests included

- uniform statistical evaluation of all tests in order to prevent response-variable differences being due to different methods,
- general statistical characteristics of the response variables "cell number", "log (cell number) ", "biomass integral", "log (biomass integral)", "mean growth rate", and "section by section growth rate" (further named "growth rate*")
- analysis of variables being discussed as validity criterion
- computation and comparison of toxicity parameters for these response variables
- interpretation of the results

Some log-transformations[8] were studied in order to test whether the respective response variables could be better used in parametric testing. Besides the properties of the response variables, the statistical evaluation included the computation of various toxicity parameters, i.e. the EC_{10}, EC_{50} and NOEC, in order to cover the requirements of most of the guidelines.

The detailed results and graphs for all measurement intervals presented in Annex B (Volume II). Detailed results of the evaluations on the basic properties of the response variable will be given in Annex A (this volume).

4.1 Methods

4.1.1 Calculation of response-variables

The biomass integral and mean growth rate were calculated according to Equations 2 and 3, respectively. The "section by section growth rate" is the growth rate during the different time periods (e.g., 0 - 24 h, 24 - 48 h, 48 - 72 h). Because the time period was 1 day throughout, Equation 3 simplifies to Equation 6.

$$\mu = \ln N_t - \ln N_{t-1} \qquad (6)$$

with:
N_t: cell number/mL at time t
N_{t-1}: cell number/mL at time t minus 1 day

[7] Note that the ratios given in Table 3 are not stated in the submitted reports, but are recalculated by the present study.
[8] The natural log was used.

Table 3: Characteristics of the test reports evaluated by the present study; in these tests the E_rC_{50} / E_bC_{50} – ratio was between 1 and 10; n.s.: not stated in the report; n.d.: not determined; Sc: *Selenastrum capricornutum*; Ss: *Scenedesmus subspicatus*; Cv: *Chlorella vulgaris*; Method: determination method for cell density; C: cell counts; E: extinction; F: fluorescence

Substance Formulation	Abbreviation (File Name)	Concentration Range [mg/L]	Method	Test Duration [hours]	Algae Species	µ of Controls 24 – 48 – 72 h	Coefficient of Variation (Cell counts, Controls) 24 – 48 – 72 h	E_rC_{50} / E_bC_{50}
Substance 19		6.25 - 100	C	96	Sc	1.28 - 1.32 - 1.34	1.24 - 3.15 - 18.31	1.06
Substance 23		0.39 - 50	F	96	Ss	1.17 - 1.11 - 1.08	5.64 - 9.12 - 5.24%	1.29
Substance 10		0.0625 - 1.0	E	96	n.s.	1.13 -1.05 - 0.89	3.09 - 7.89 - 6.51%	1.34
Substance 2		0.046 – 1.0	C	72	n.s.	1.58 - 1.55 - 1.48	20.61 - 22.83 - 18.59%	1.92
Substance 7		10 - 100	C	72	n.s.	0.36 - 1.28 - 1.20	24.19 - 44.98 - 21.04%	1.98
Substance 25		0.1 - 1.6	C	72	Sc	0.44 – 0.94 - 1.68	154.92 - 85.6 - 38.79%	2.0
Substance 8		0.0075-0.210	C	120	Sc	1.56 - 1.53 - 1.46	34.64 - 25.42 - 8.77%	2.45
Substance 31		0.013 - 0.402	C	72	Sc	1.54 - 1.49 - 1.45	7.64 - 8.28 - 11.32%	2.50
Substance 33		3.125 - 50	E	96	Ss	1.38 - 1.24 - 1.13	30.13 - 25.66 - 9.59%	2.50
Substance 17		0.018 - 0.56	C	72	Ss	1.59 - 1.61 - 1.53	18.44 - 47.93 - 52.12%	3.14 (93 h)
Substance 16		0.1 - 31.623	E	93	Ss	1.41 - 1.40 - 1.41	7.31 - 6.73 - 3.12%	3.23
Substance 6		0.039 - 40		72	n.s.	1.18 - 0.93 - 1.06	5.43 - 5.54 - 0.35%	5.18
Substance 26		1 - 56	C	72	n.s.	1.86 – 2.08 - 1.76	47.13 - 79.87 - 79.20%	5.50
Substance 5		1.6 - 400	C	96	n.s.	0.96 - 0.70 - 1.09	10.96 - 12.91 - 12.72	5.90
Substance 22		0.025 - 6.4	C	120	Sc	1.68 – 1.62 - 1.64	5.50 - 10.14 - 6.61%	7.04
Substance 37		0.003 - 1.0	C		Ss	1.18 - 1.25 - 0.98	7.88 - 16.78 - 7.33%	7.97
Substance 36		0.003 - 1.0	C	96	Ss	2.35 - 1.51 - 1.22	14.64 - 20.96 - 45.36%	9.50

4.1.2 Statistical properties of the response variables

The normal distribution and variance homogeneity has to be checked, in case the NOEC is determined by a statistical test. If both prerequisites are fulfilled the beneficial parametric test procedures (e.g., ANOVA procedures: Dunnett's test, Williams test) can be applied, which are more powerful than non-parametric ones (e.g., Bonferroni U-test; see below).

The normal distribution of the test data sets was examined using the R/s-Test (Sachs 1992). In contrast to more frequently used Kolmogoroff-Smirnov-test, this test can be performed at sample sizes smaller than 4, which was commonly the case with treatments of the AGIT. The R/s-test uses only two statistical measures, the range, R, and the standard deviation, s, the ratio of which ideally is about 3. The computed values R/s for each treatment and control are compared with the margins of R/s, tabulated by Sachs (1992). These margins give a range, in which the sample R/s may vary by chance and which is in correspondence with the normal distribution hypothesis. In addition, replicate values for %inhibition relative to the control of every response variable were generated, using every data pair which could be formed from the number of control replicates (n_c) and replicates of a treatment (n_t). The number of %inhibition replicates ($N = n_c * n_t$) was sufficiently to apply the Kolmogorroff-Smirnov test (modification after Lilliefors (1967)) for normal distribution.

The variance homogeneity of the original test data sets was examined using Cochran's test and - in case of %inhibitions at higher replicate numbers - Bartlett's test procedure (Sachs 1992).

Table 4: Characteristics of the test reports evaluated by the present study; in these tests the E_rC_{50} / E_bC_{50} – ratio was either below 1 or greater than 10 (rest see Table 3); grey: extreme E_rC_{50}/E_bC_{50}–ratios

Substance Formulation	Concentration Range [mg/L]	Method	Test Duration [hours]	Algae Species	µ of Controls 24 – 48 – 72 h	Coefficient of Variation (Cell counts, Controls) 24 – 48 – 72 h	E_rC_{50} / E_bC_{50}
Substance 9	1 - 64	C	72	Sc	1.36 - 1.41 - 1.47	5.21 - 14.29 - 15.93%	n.d.
Substance 32	0.18 - 3.2	C	72	Sc	1.92 - 1.79 - 1.61	5.71 - 5.54 - 11.01%	n.d.
Substance 21	0.01 - 100	C	72	Ss	0.37 - 1.1 - 1.2	4.88 - 5.24% - n.d.	0.63
Substance 20	0.01 - 100	C	72	Sc	0.76 - 1.18 - 1.25	6.84 - 11.59 - 16.97%	0.71 (96 h)
Substance 24	0.054 - 1.5	C	120	Sc	1.14 – 0.86 - 1.10	16.43 - 19.89 - 10.81%	11.4
Substance 34	0.30 - 30	C	96	n.s.	1.90 - 1.72 - 1.78	5.62 - 2.27 - 1.40%	13.1
Substance 12	0.001 - 0.1	C	96	Ss	2.23- 1.74- 1.51	29.74 - 26.79 - 20.51%	18.8
Substance 11	5.6 – 100	C	72	Sc	1.82 - 1.94 - 1.90	6.17 - 6.95 - 2.98%	23.5
Substance 38	0.012 - 12.0	C	72	n.s.	0.99 - 1.3 - 1.55	40.08 - 35.24 - 33.58%	24.0 (48 h)
Substance 4	0.063 - 2	C	96	n.s.	0.26 - 0.61 - 1.64	37.54 - 32.45 - 7.08 %	33.6.
Substance 27	0.01 - 0.16	E	120	n.s.	0.89 - 0.91 - 0.75	0.86 - 1.83 - 1.13%	39.0
Substance 29	0.36 - 1200	E	72	Sc	1.01 - 1.48 - 1.51	5.26 - 3.65 - 3.66%	41.4
Substance 28	0.32 - 320	C	72	Sc	0.87 - 1.56 - 1.52	9.35 - 4.53 - 7.39%	44.1
Substance 13	0.1 – 22	C	72	n.s.	1.69- 1.55 - 1.12	18.46 - 18.20 - 6.74%	50.0
Substance 35	0.47 - 190	C	72	Sc	0.96 - 1.22 - 1.38	n.s. - 44.61 - 3.92%	89.8.
Substance 15	0.158 - 500	C	72	n.s.	1.2 - 1.04 - 1.43	20.98 - 15.93 - 3.37%	150.1
Substance 1	1 - 100	C	72	Ss	1.82 - 1.81 - 1.66	32.67 - 10.39 - 14.28 %	218
Substance 18	0.0125 - 0.8	E	72	n.s.	0.74 - 0.94 - 0.99	6.48 - 22.34 - 9.88%	240
Substance 14	0.24 - 5	E	72	n.s.	1.68 - 1.42 - 1.24	10.62 - 48.39 - 43.30%	480
Substance 30	0.191 - 12.516	C	72	Cv	<0 - 1.12 - 1.45	24.47 - 36.14 - 16.01%	1084

4.1.3 Determination of the NOEC (threshold concentration)

The NOEC was determined by Dunnett's test as described above (Section 2.5.1). In addition, to compare different test methods, Williams test and Bonferroni U-test, the non-parametric alternative, was applied (Holm 1979). These tests are multiple and – as described above - the significance level (α-error) is adjusted to the whole experiment (per experiment error). The null hypothesis, H_0: $\mu_0 = \mu_1 = \mu_2 = ... = \mu_n$, states that the observed differences are by chance rather than are due to the test substance. Critical margins have been tabulated for Dunnett's test (Dunnett 1955, 1964) and for the Bonferroni-U-test by Lienert (1975). The significance level was set to $\alpha = 0.05$ (5%; one-sided smaller). For Dunnett's and Williams test, the pooled variance estimate, obtained with an one-way ANOVA, was used.

If a solvent control was investigated in an AGIT, the difference between control and solvent control was tested using STUDENT t-test (Sachs 1992). If these proved to be equal, both controls were pooled, otherwise the solvent control served as reference for the NOEC determination.

4.1.4 Computation of effective concentrations ECx

The values of the response variables of all time intervals were used to determine the magnitude of inhibition as caused by the test substances. The percentage inhibition relative to the control was calculated by Equation 5.

The response (percentage) plotted over the log concentration results in the so called substance-specific concentration-response curve. In the ideal case, with the AGIT response variables it follows the cumulative normal distribution. Whenever possible, the normal sigmoid was fitted to the data by probit analysis. This was done by means of iterative regression analysis (maximum likelihood method, Finney 1978). Values ≤0% or ≥100% are not compatible with the normal distribution and, therefore, were principally excluded from evaluation.

If the probit analysis was impossible, the Moving Averages approach was performed. Moving averages were calculated from the response-variable values and the concentrations. Thereafter, the EC_{50} is interpolated from values in the nearest neighbourhood of the EC_{50}. The method requires no assumption concerning the type of statistical distribution of the data. Averages over three values are commonly computed and thus are given as results, which in these cases are marked with an “m”.

4.1.5 Software

For the computations existing validated PC-software (EASY ASSAY Algae Growth Inhibition (AGI), EASY ASSAY Multiple Testing (MT)), developed by the present authors was adapted to the more comprehensive evaluation conducted by the present study. AGI was completed by the evaluation of cell counts, section by section growth rates and computation of the EC_{10}, EC_{20}, EC_{50} and NOEC for all of the response variables. Both programs were enhanced to perform serial computation and produce output of results for further evaluation and graphing by MS EXCEL 97. The detailed results are given in Annex B (Volume II) and C (Volume III).

4.2 Detailed description of the growth course

This section introduces the tests, performed for the various substances, by means of their growth curves together with a brief description of the growth pattern during the experimental time. The growth curves will be assigned to various categories or characteristic scenarios, reflecting various experimental problems and properties of the test substance (Chapter 5). The growth curves will be presented in alphabetical order.

4.2.1 Substance 1

In the control the growth was nearly exponential until 72 h. There was some inhibition of growth at middle concentrations. A marked inhibition occurred at higher concentrations between 24 and 48 h. Generally after 72 the growth rate was reduced at all concentration including the control, probably due to some limitation (e.g., nutrients, carbon dioxide; Figure 4)

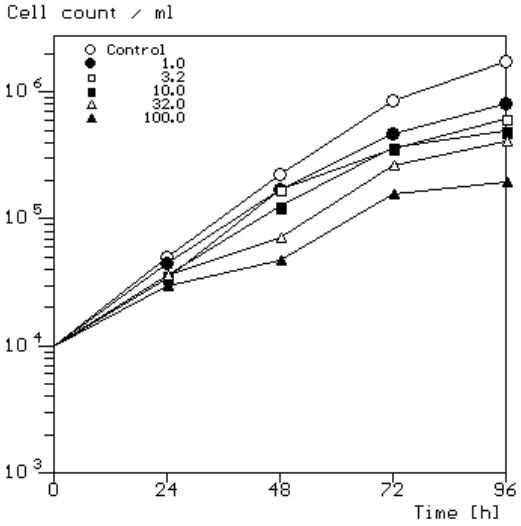


Figure 4

4.2.2 Substance 2

The growth was nearly exponential in the control and lower concentrations during the experimental period of 72 h. There was a slight growth promotion at the lowest test concentration and a slight inhibition at concentrations below the highest one. A strong growth inhibition was to be observed at the highest test concentration (Figure 5).

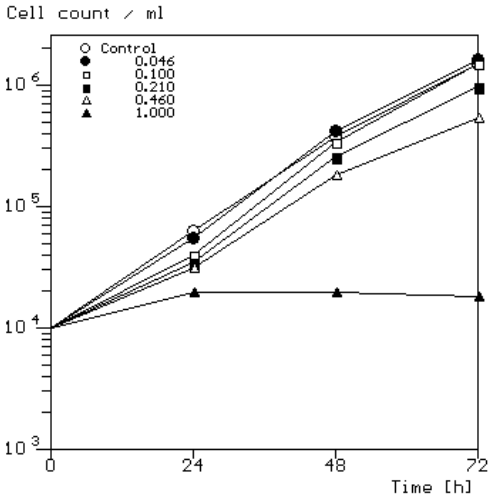


Figure 5

4.2.3 Substance 3, 4

Exponential growth in the control was lacking. All treatments and the control showed a lag phase until 48 h. At 24 h, a transient effect was to be observed in the highest two concentrations. After 72 h the growth rate was reduced in all treatments and the control (Figure 6). This substance was evaluated twice: with particles (3) and without particles (4).

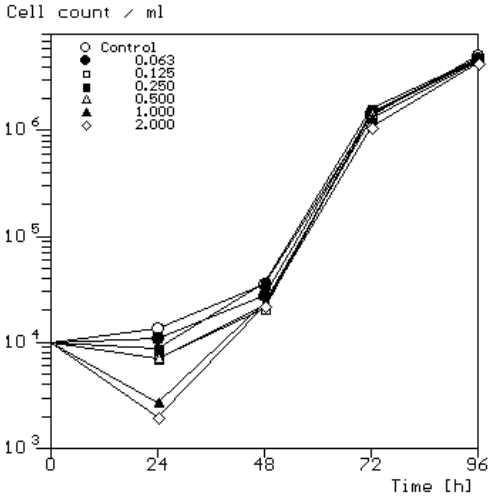


Figure 6

4.2.4 Substance 5

As with the previous test all of the test cultures showed an initial lag-phase until 48 h and thus no exponential growth occurred throughout the test period. There might be an interim inhibiting effect between 48 and 72 h or a prolonged lag-phase in the highest three concentration. After 72 h the growth appears to proceed uninhibitedly in all concentrations and the control (Figure 7).

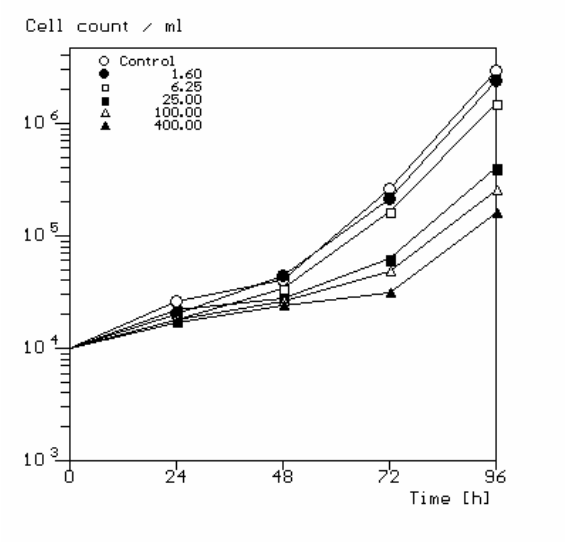


Figure 7

4.2.5 Substance 6

The growth proceeded nearly exponential at moderate rates in all treatments, except that in three of the test cultures an intermediate reduction of the growth rate was to be observed. There was a gradual inhibition due to the test substance. The inhibitory effect appears to become smaller after 48 h – at least in the highest test concentration (Figure 8).

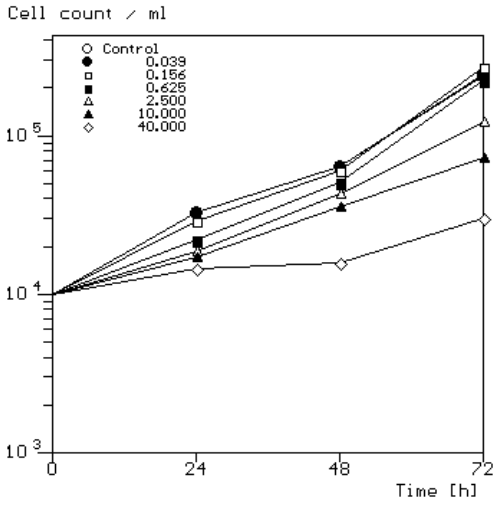


Figure 8

4.2.6 Substance 7

Overall, the growth curves show substantial variability, which obscures probably the underlying exponential growth pattern and was somewhat disturbed due to variability. Strong inhibition occurred in the highest two concentrations. In the highest concentration cell death could be the reason for the decline (Figure 9).

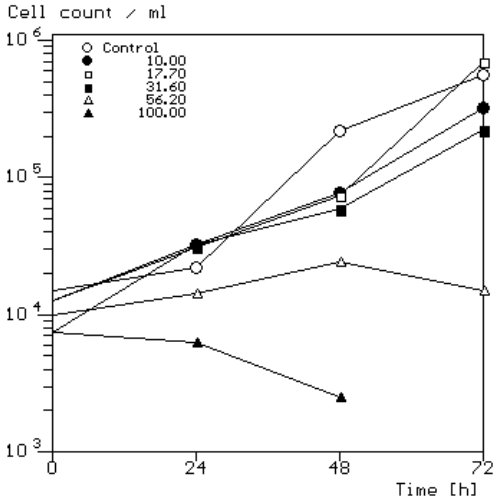


Figure 9

4.2.7 Substance 8

Exponential growth proceeded until 72 h, thereafter the growth slowed down in the control and all of the test concentrations. Test substance effects became stronger after a time-lag (due to bioaccumulation or interference with nutrients?) (Figure 10).

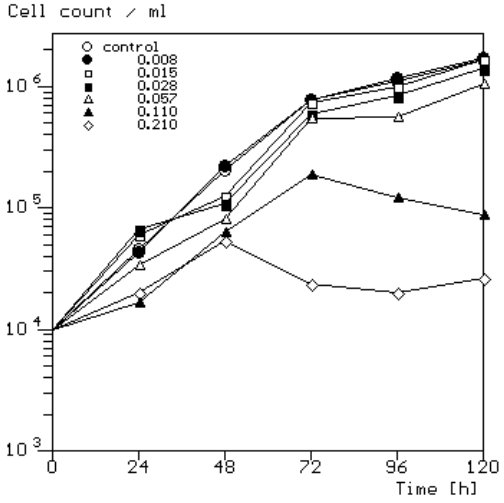


Figure 10

4.2.8 Substance 9

All test cultures showed exponential growth. The test substance had a slight promoting effect at lower concentrations and only a slight inhibiting effect at higher concentrations (Figure 11).

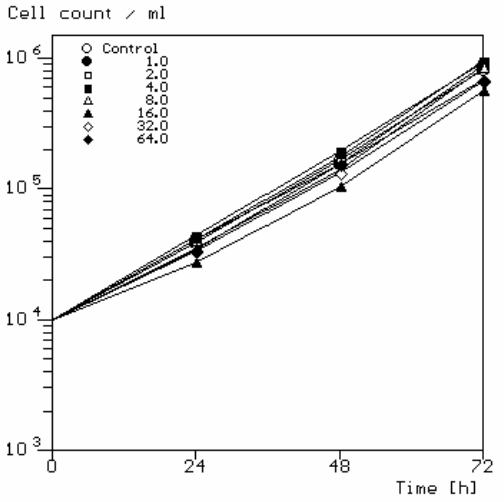


Figure 11

4.2.9 Substance 10

A slight decrease in the growth rate was found after 48 h. Marked effects were visible in the two highest concentrations after 48 h (Figure 12).

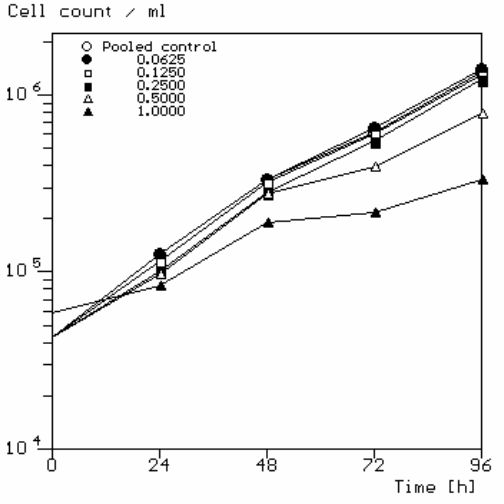


Figure 12

4.2.10 Substance 11

In the control and lower concentrations the growth was nearly exponential. There was a slight growth promotion by the lowest concentration. The two highest concentrations caused a drastic decline in cell number (cell death) until 48 h. Thereafter growth started again (Figure 13).

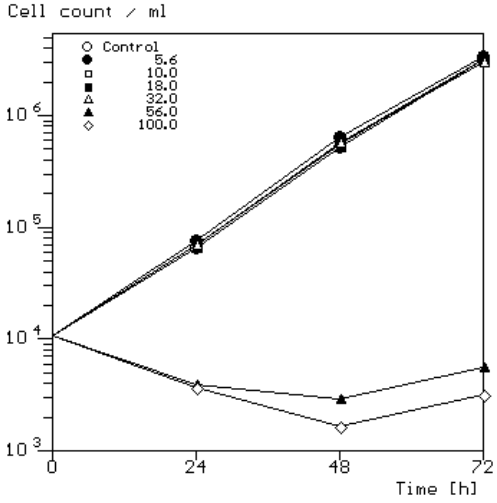


Figure 13

4.2.11 Substance 12

Except for the control and the highest concentration at 0-24 h, there was nearly exponential growth in all concentrations. The growth rate of the control culture at 0-24h was unusually high (μ = 2.23 d^{-1}), so that probably an error occurred in setting the starting cell density in the control (> 10^4 Cells/mL) (Figure 14).

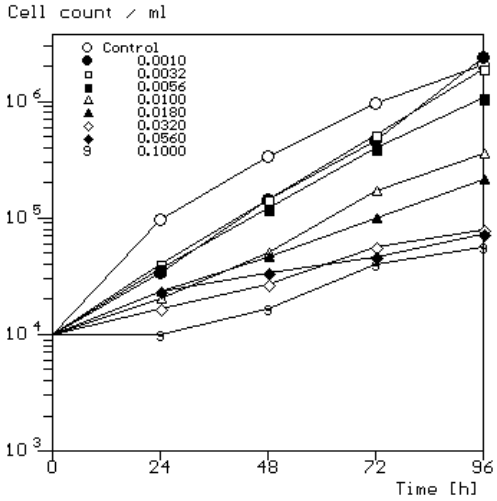


Figure 14

4.2.12 Substance 13

In all test cultures the growth was nearly exponential until 48 h, thereafter growth was reduced in the control and lower concentrations (Figure 15). This could be due to the relatively high start cell density.

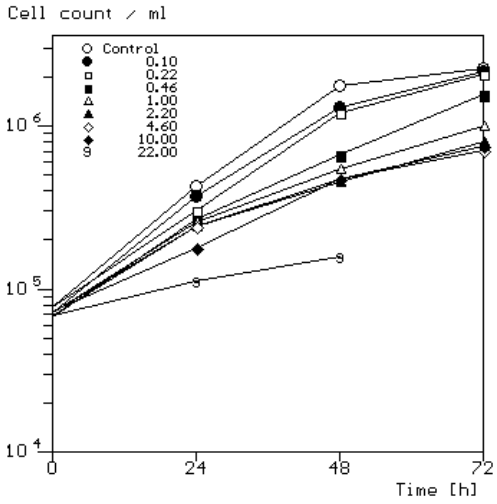


Figure 15

4.2.13 Substance 14

In all concentrations the growth was nearly exponential after 24 h. Among possible reasons for the steeper slope at 0 – 24 h are error in determination of the start density (measured by extinction), limitation of a nutrient, and reduction in light intensity or temperature (Figure 16).

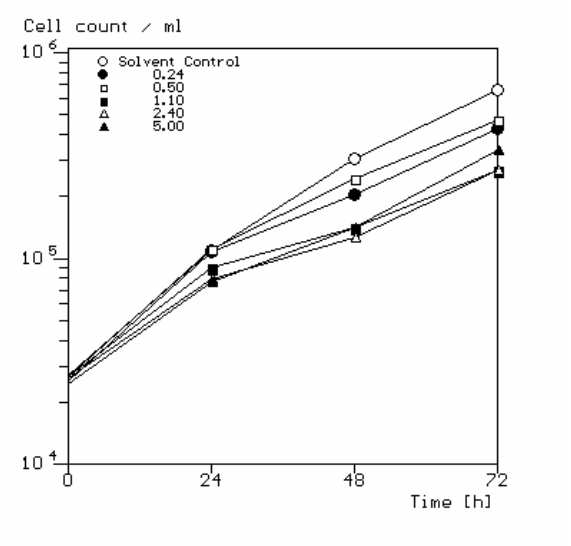


Figure 16

4.2.14 Substance 15

At 24 to 48 h, a slowing down of the growth occurred. The test substance obviously affected the growth before 48 h, thereafter about the same growth rates were to be observed in most concentrations (Figure 17).

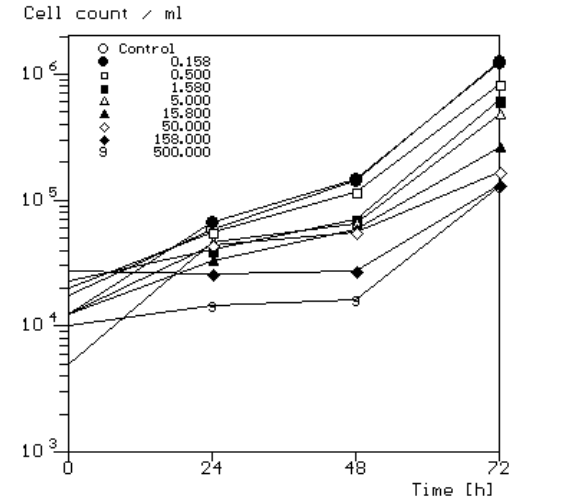


Figure 17

4.2.15 Substance 16

Except for the highest concentration, the growth was nearly exponential at low to zero effects of the test substance. In the highest concentration the starting cell density might have been incorrectly set, which is indicated by an unusually high starting growth rate of $\mu(0\text{-}24h) = 3.29\ d^{-1}$. Thereafter no positive growth was to be observed (Figure 18).

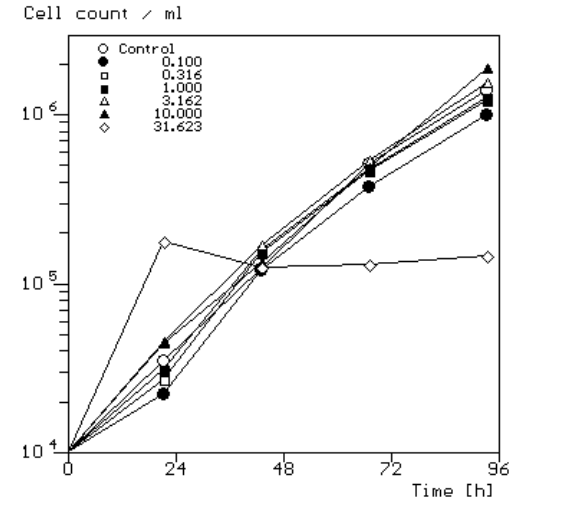


Figure 18

4.2.16 Substance 17

There was nearly exponential growth in the control and all of the concentrations. The selection of test concentrations appears appropriate (Figure 19).

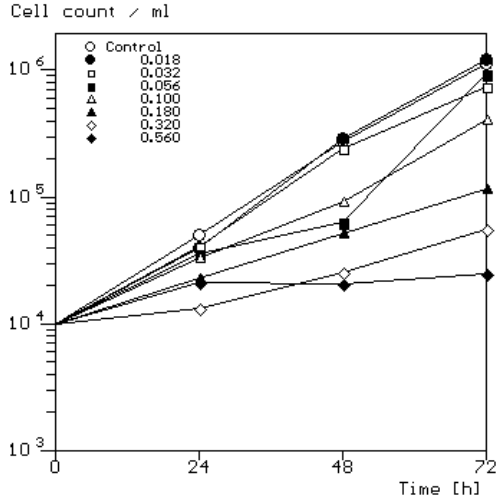


Figure 19

4.2.17 Substance 18

The growth pattern was variable. In the control and some concentrations exponential growth set in after a lag-phase (0-24 h). Obviously, a recovery occurred after 48 h (Figure 20).

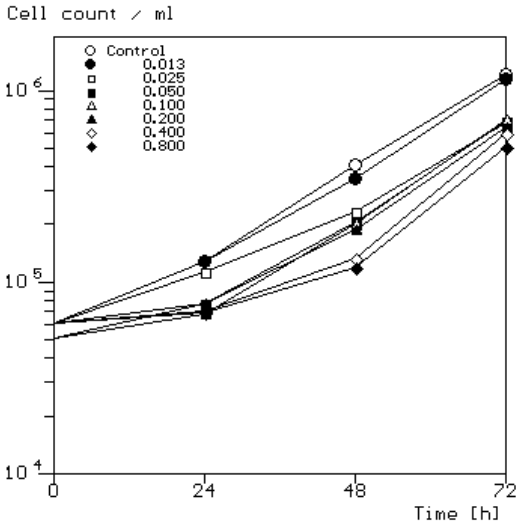


Figure 20

4.2.18 Substance 19

All test cultures exhibited exponential growth. The growth was slightly promoted by the two lowest concentrations (Figure 21).

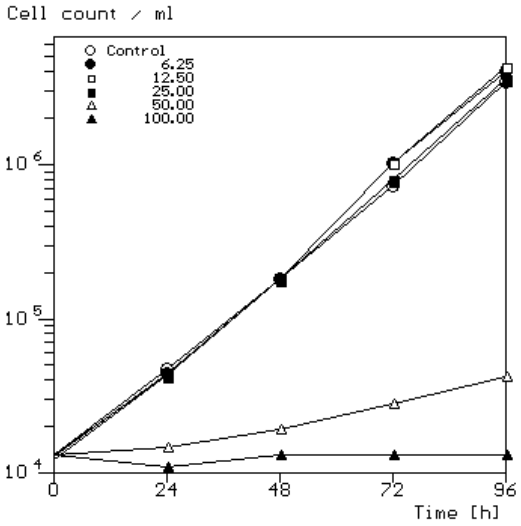


Figure 21

4.2.19 Substance 20

All test cultures grew nearly exponential at least after 24 h, while the growth was promoted by the test substance except for the highest concentration (Figure 22).

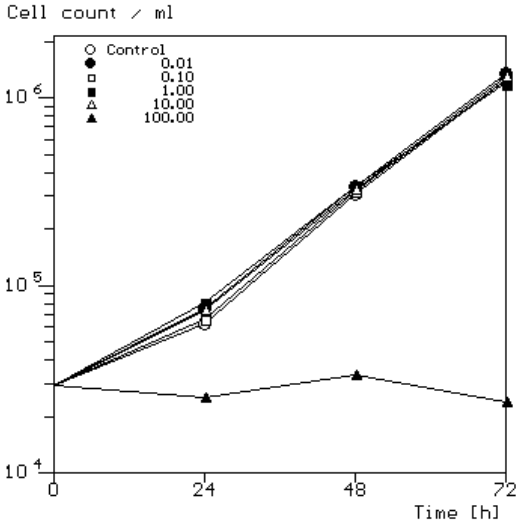


Figure 22

4.2.20 Substance 21

After a lag-phase with cell death in the highest concentration until 24 h, exponential growth was to be observed throughoutly (Figure 23).

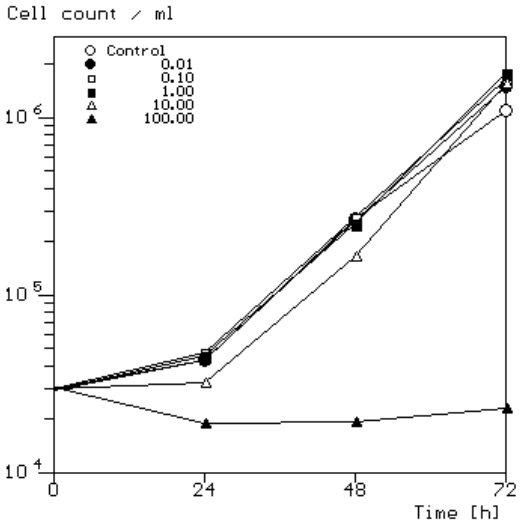


Figure 23

4.2.21 Substance 22

The growth was found to be exponential until 96 h, thereafter growth was slightly reduced. The concentration range appears appropriate (Figure 24).

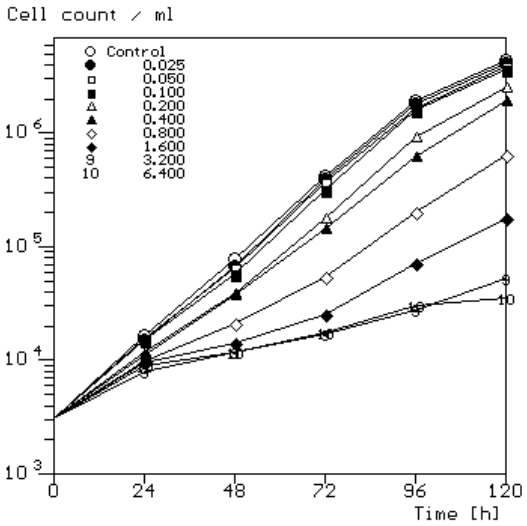


Figure 24

4.2.22 Substance 23

The control culture and those in the lower concentrations showed exponential growth all the time. Exponential growth was also seen in the higher concentrations after a lag-phase. It appears that there was an error in setting the start density (Figure 25).

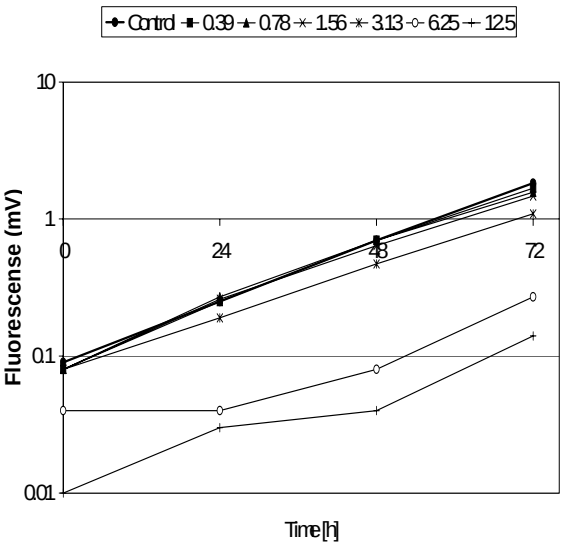


Figure 25

4.2.23 Substance 24

There was nearly exponential growth for a longer period in the control and the lower concentrations. Interference was probably due to an error in determining the starting cell density, some inconstancies in the culture conditions (24 – 48 h, light, temperature), and nutrient limitation (after 96 h) (Figure 26).

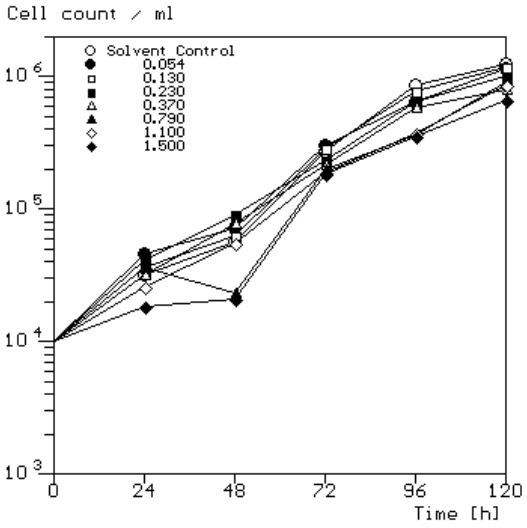


Figure 26

4.2.24 Substance 25

There was a lag-phase until 24 h or an error in starting cell densities, thereafter growth proceeded exponentially in some cases or variability led to a differing growth pattern (Figure 27).

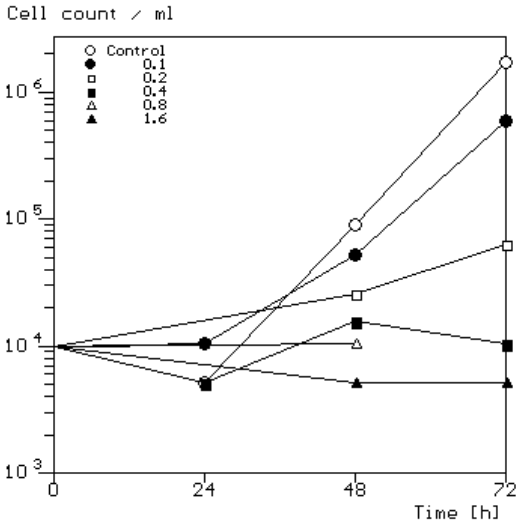


Figure 27

4.2.25 Substance 26

Until 48h the growth was nearly exponential, thereafter it slowed down in the control and lower concentrations. In the two highest concentration the cell density increased initially, before it was reduced to a lower value (Figure 28).

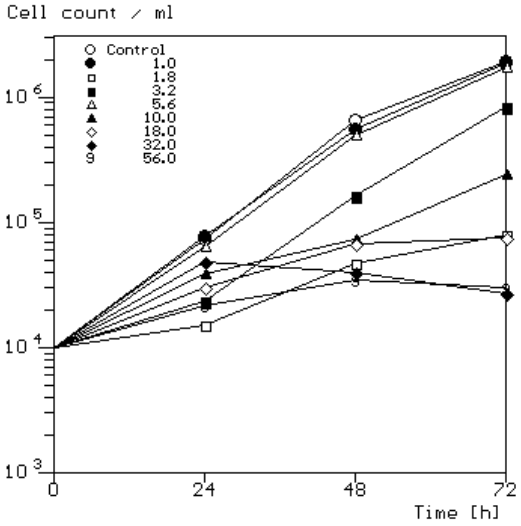


Figure 28

4.2.26 Substance 27

The growth was nearly exponential until 96 h, also when it was negative. Thereafter it slowed down in the control and the two lowest concentrations, obviously due to some limitation. There was only one concentration with effects between 0 and 100% (Figure 29).

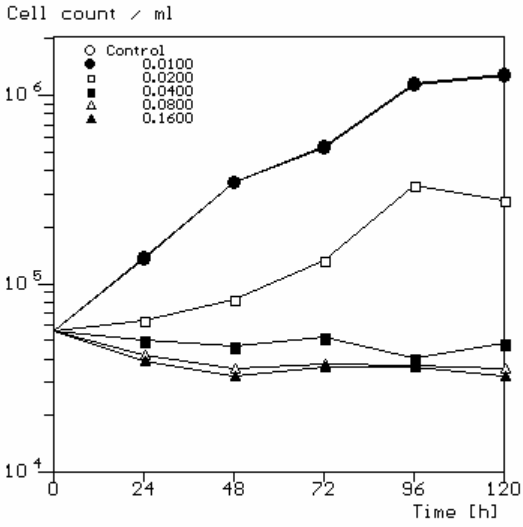


Figure 29

4.2.27 Substance 28

The growth curves are characterised by an initial lag-phase up to 24 h and a decline after 48 h (Figure 30).

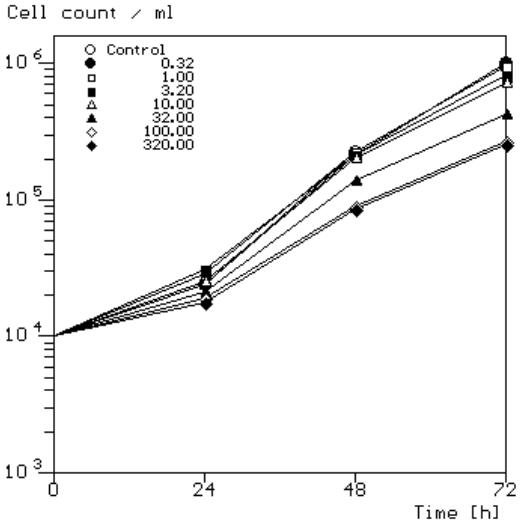


Figure 30

4.2.28 Substance 30

After a lag-phase with zero growth until 24 h, the test cultures grew exponentially with a growth promotion in the lowest concentration (Figure 31).

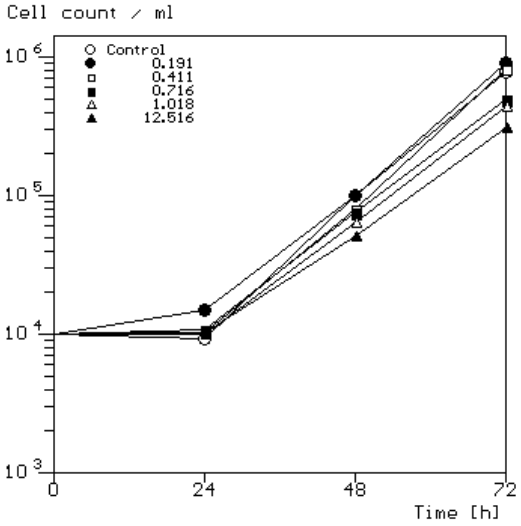


Figure 31

4.2.29 Substance 31

The control and the lowest concentrations as well as the remaining concentrations until 48 h showed good correspondence with exponential growth. In two middle-range concentrations growth recovered after 48 h (Figure 32).

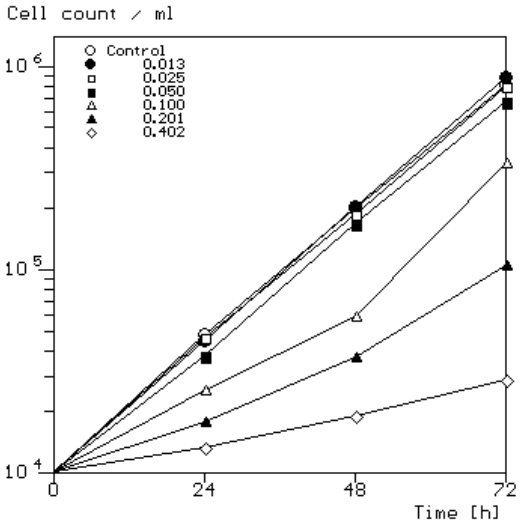


Figure 32

4.2.30 Substance 32

There was nearly exponential growth throughoutly with small effects of the test substance (Figure 33).

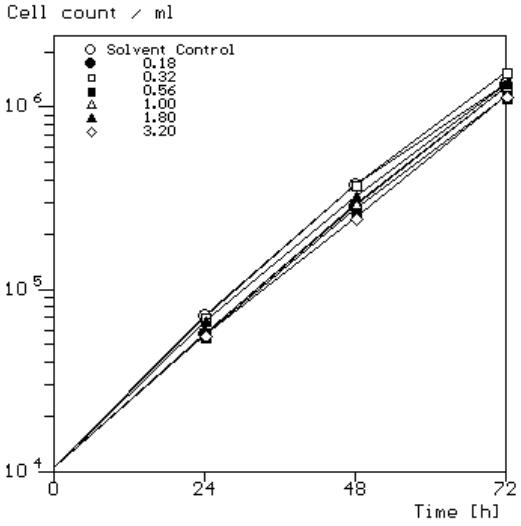


Figure 33

4.2.31 Substance 33

Growth proved to be nearly exponential in the control and lower concentrations until 72 h, before it slowed down to some extent. At higher concentrations, a marked effect of the test substance was obvious until 24 h. Thereafter growth recovered (Figure 34).

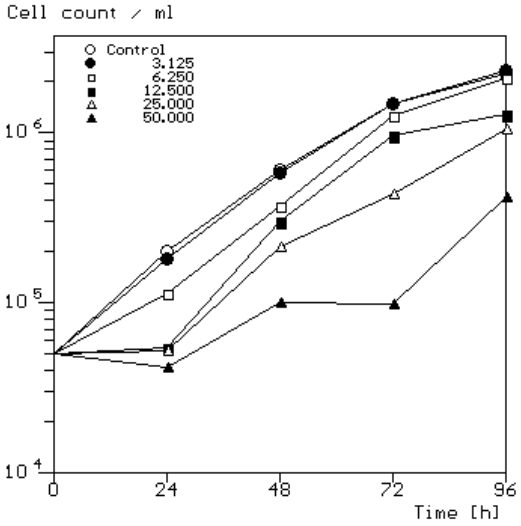


Figure 34

4.2.32 Substance 34

The overall impression is that growth proceeded nearly exponential, although the variability at lower densities appears to interfere with this impression. There could be a lag-phase in the treatments and growth slowed own after 72 h (Figure 35).

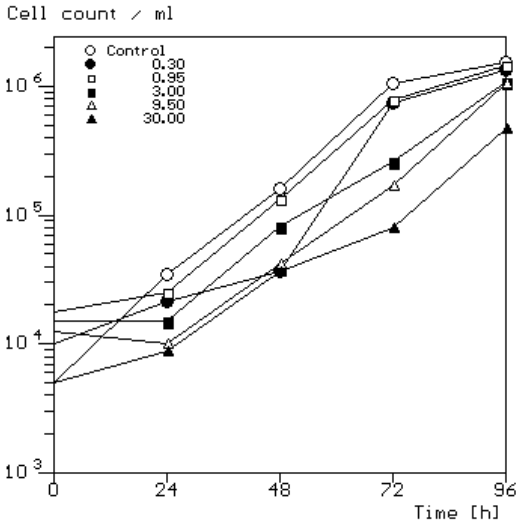


Figure 35

4.2.33 Substance 35

The exponential uninhibited growth started after 24 h. Obviously there was a lag-phase and a temporary effect of the test substance before 24 h (Figure 36).

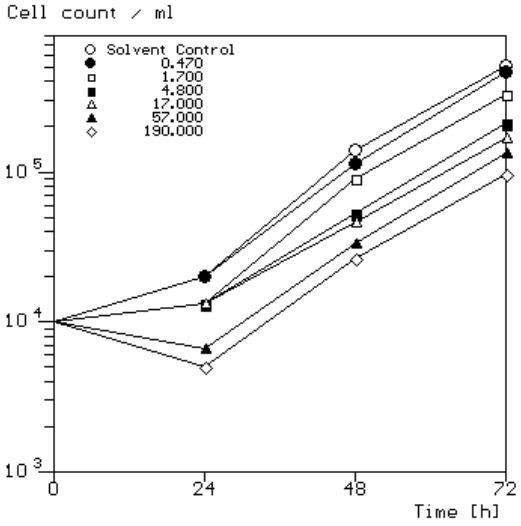


Figure 36

4.2.34 Substance 29

After a lag-phase until 24 h, where no effects of the test substance were visible, the growth proceeded exponentially and was gradually inhibited by the test substance (Figure 37).

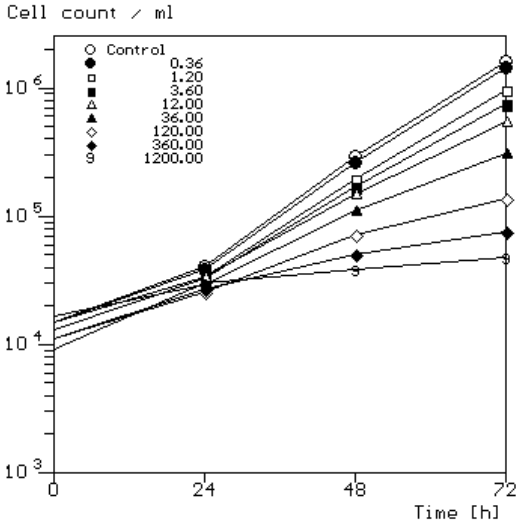


Figure 37

4.2.35 Substance 36

Obviously due to an experimental error in determination of the start density, an unusual high growth rate of $\mu > 2.2\ d^{-1}$ was reported for the first 24 h. Thereafter, exponential growth lasted until 72 h and then was slowed down by some limiting factor (Figure 38).

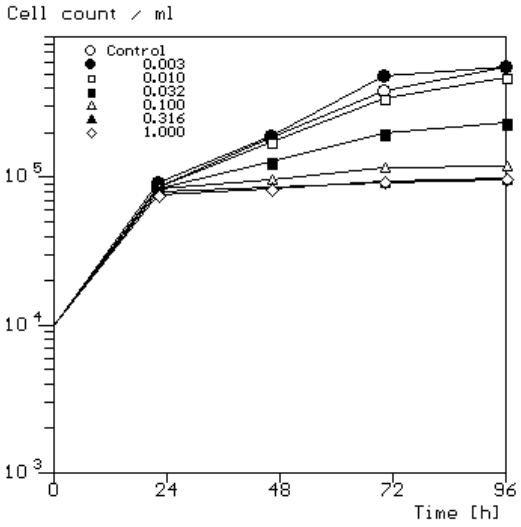


Figure 38

4.2.36 Substance 37

The test cultures apparently grew to a limit, which was reached at 48 h in the control and at 72 h in the lower concentrations. At higher concentrations, the test substance increasingly affected the growth (Figure 39).

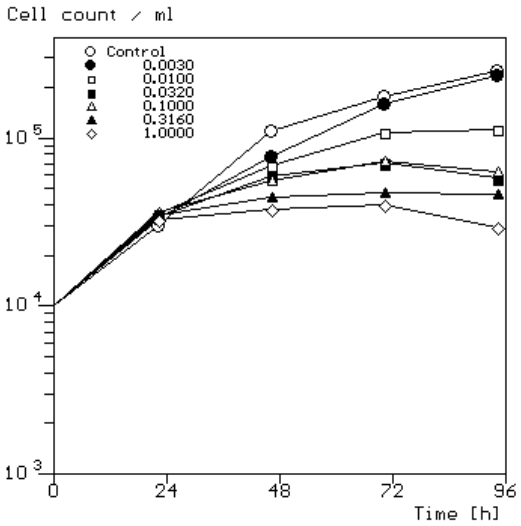


Figure 39

4.2.37 Substance 38

Exponential growth was found in the control and the lower concentrations. A promoting effect of the test substance was observed at the lowest concentration and an inhibiting one at higher concentrations until 24 h (Figure 40).

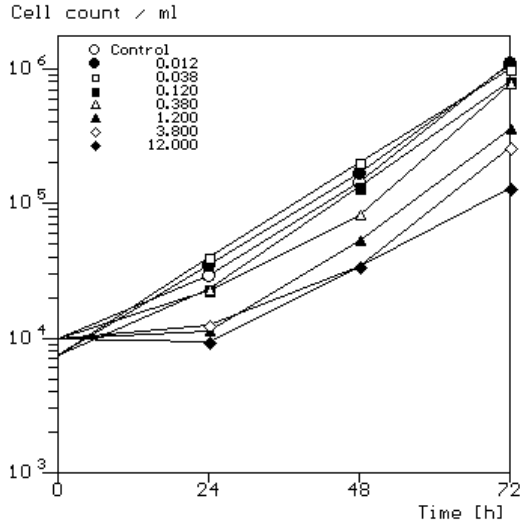


Figure 40

4.3 Interpretation of the growth pattern by characteristic scenarios

4.3.1 Introduction

It could be shown, that the growth pattern differed among the various substances. For an appropriate assessment of the true toxicity of a test substance it is inevitable to distinguish between growth patterns caused by the properties of the test substance and those due to obvious experimental shortcomings or mistakes in the evaluation of the results. Before examining the effects of the growth pattern on the response variable relations, it appeared appropriate to lead the many observed patterns back to fewer basic ones. Twelve basic scenarios were identified, to which nearly all of the growth patterns of the growth curves from the submitted tests could be assigned – more or less strictly. In some of the submitted cases, growth curves could be observed in which different scenarios were intermixed. Every scenario will be exemplified by a graph, stemming from a simulated algae test, which will be used to perform a generalised evaluation of response-variable relations (see Chapter 5). From many empirical examples and theoretical considerations, it is reasonable to accept the exponential growth as the basic pattern in unicellular micro-organisms, provided no limitations come into play. Therefore, the exponential growth was underlying in all example scenarios shown below. Deviations from the ideal exponential growth curves were superimposed to the exponential growth by adding promoting and inhibiting functional terms, sometimes constantly acting or changing over time. This is justified since growth rates behave in an additive manner.

4.3.2 Basic growth pattern: the exponential growth

The exponential growth (Scenario 1) should prevail at least in the control for a certain period of time (e.g., at least until 72 h), provided the test conditions are appropriate (e.g. illumination, temperature, culture medium). If the toxic action of a test substance remains constant during the test period and none of the modifying factors on the control or treatments are acting, the growth proceeds exponentially in all treatments over time. This is exemplified by Figure 41.

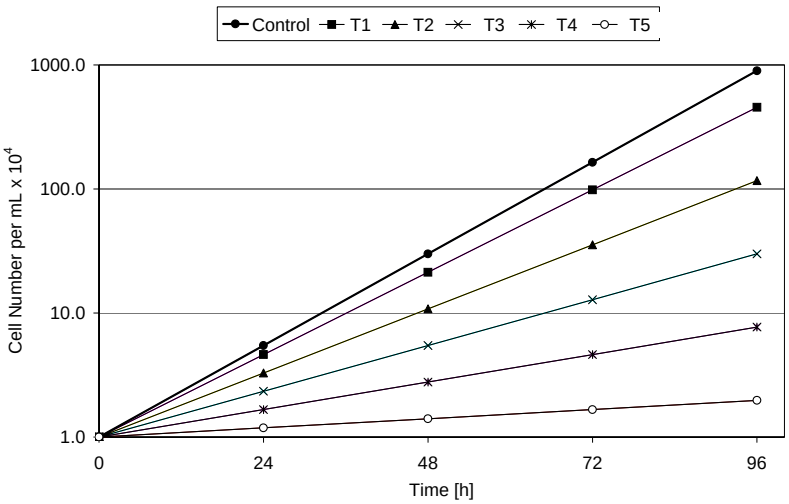


Figure 41: Exponential growth of treated and non-treated algae cultures throughout the experiment. Note that this is an ideal case which assumes immediate and continuous toxic action as well as absence of nutrient limitation.

This growth pattern was realised nearly perfectly in Substance 2, Substance 9, Substance 20, Substance 31, and Substance 32. In many of the remaining substances part of the control and treatment showed strictly exponential growth or this type of growth was maintained in the initial experimental period, before some limitation was apparent. Among these were Substance 1, Substance 6, Substance 11, Substance 12, Substance 13, Substance 14, Substance 16, Substance 19, Substance 22, Substance 23, Substance 24, Substance 25, Substance 26, Substance 27, Substance 33, and Substance 38.

4.3.3 Growth modification due to experimental errors and shortcomings

Various types of experimental errors arise from non-constant conditions during growing (light, temperature), growth limitation (nutrient or carbon dioxide depletion), use of algae from non-log-phase stock cultures, and simply a false assumption of the starting cell density due to an error. Example scenarios with the respective growth curves are shown below. In addition, these scenarios can occur in various combinations.

4.3.3.1 Inconstant temperature and light conditions (Scenario 2)

This type of experimental shortcomings evokes a simultaneous and temporary increase or decrease in the slope of the growth curves (growth rate) of <u>all treatments including the control</u> as exemplified in Figure 42. This pattern applies also for single treatments, in case their test vessels had been placed temporarily in sub-optimal temperature or light conditions.

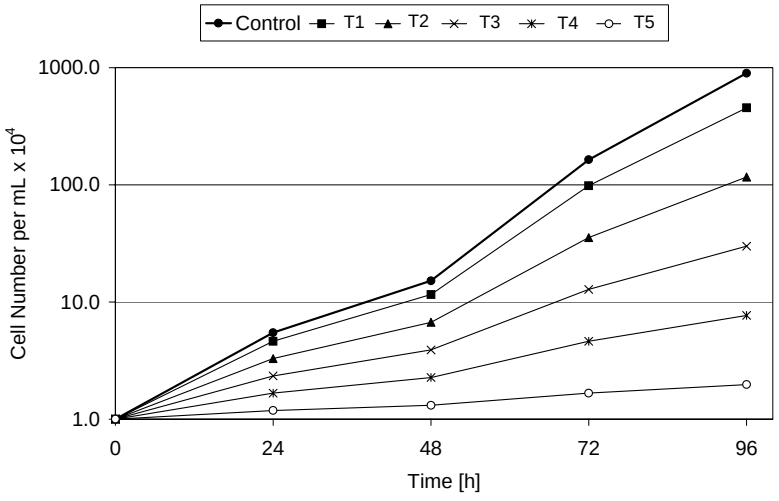


Figure 42: Temporary reduction of the growth rate of algae cultures as caused by a temporary drop in temperature or light intensity

With Substance 6, Substance 14, Substance 15, and Substance 24 such sort of growth pattern was to be observed. Note that in the fast growing treatments this effect is more pronounced. It is hardly possible that the tested substances were responsible for the pattern since it was also realised in the control.

4.3.3.2 Depletion of essential resources (Scenario 3)

Depending on the culture medium used and the test duration, essential nutrients (e.g. phosphate, carbon dioxide) can be used up and limit the growth rate of the culture. As a rule, fast growing cultures, such as the control or slightly inhibited or promoted treatments, are more strongly affected (Figure 43). A similar pattern would be expected, if the test substance interferes with nutrients, making them unavailable for the algae. Here especially the algae from higher test concentrations would be increasingly affected. This growth pattern would be hardly to distinguish from increasing toxicity of the test substance. A respective growth pattern is discussed with Scenario 10 (Section 4.3.4.3).

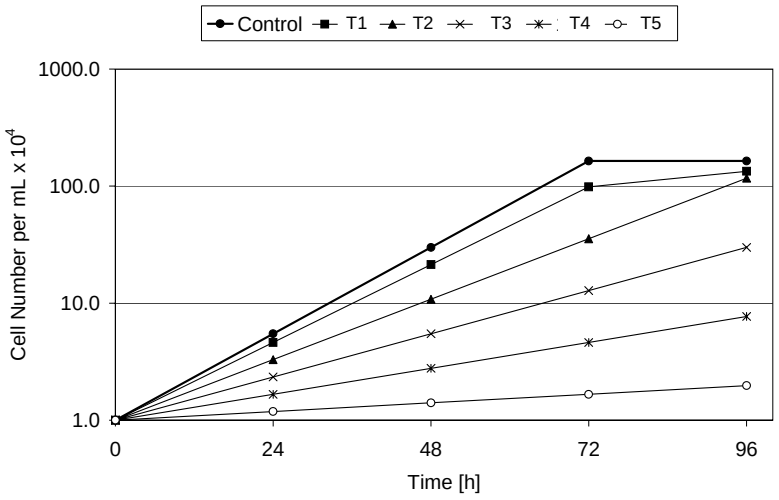


Figure 43: Reduction of the growth of algae cultures as caused by a nutrient or carbon dioxide depletion during the final phase of the experiment. Note that the fast growing treatments are more strongly affected.

Model tests for such sort of growth pattern are those with Substance 13, Substance 17, and Substance 22, but growth patterns suggesting some limitation were frequently found also in other substances: Substance 1, Substance 4, Substance 8, Substance 10, Substance 12, Substance 24, Substance 26, Substance 27, Substance 28, Substance 34, Substance 33, Substance 35, Substance 36, and Substance 37.

Although in some cases the limitation was visible already after 48 h, the majority of such sort of growth pattern was to be observed at test durations greater than 72 h, such as exemplified in Figure 50. This indicates that test durations shorter than 96 h is more appropriate.

4.3.3.3 Initial lag-phase (Scenario 4)

The initial lag-phase – in case also present in the control – indicates that either the inoculum was taken from a non-log-phase stock culture or other test conditions (e.g., culture medium, temperature, and light intensity/quality) differed in the stock and test cultures, so that the algae needed some adaptation time. Symptomatic for this type of growth patters is a retardation of growth in all of the test cultures. Figure 44 gives the pattern, caused by a lag-phase without cell loss in the treated cultures.

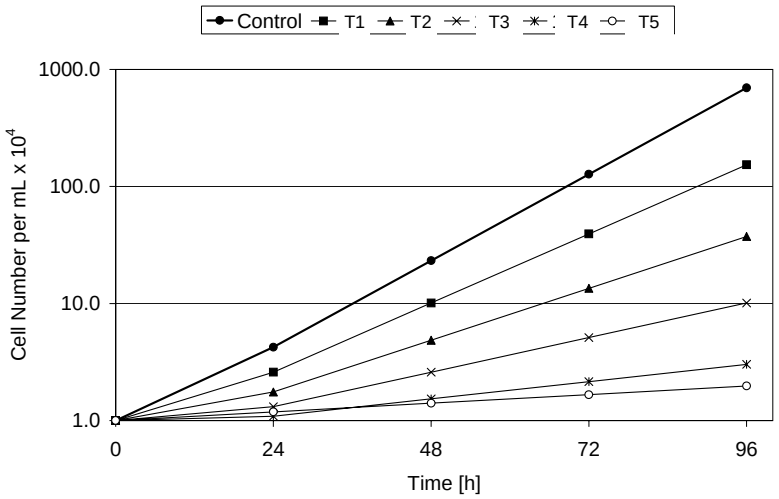


Figure 44: Growth delay of algae cultures during an initial lag-phase without algaecidal effects.

Such sort of growth pattern was found with Substance 5, Substance 18 Test 1, Substance 28, Substance 30 Test 1, and Substance 29.

4.3.3.4 Initial lag-phase with algaeicidal effects (Scenario 5)

A variant of the previous scenario is presented by Figure 45. Depending on the mode or strength of toxic action, the cell density can be reduced by mortality, which is visible in a decline of cell density below the starting density.

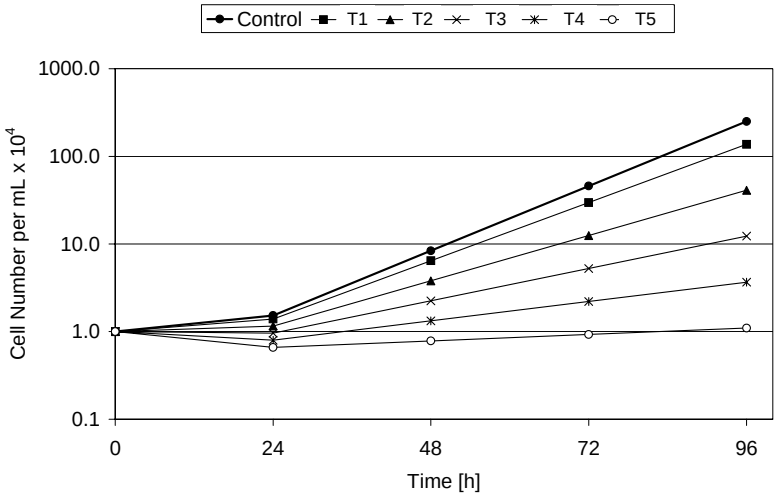


Figure 45: Growth delay of algae cultures during an initial lag phase with some algaecidal effect at higher test concentrations.

Such type of effect was observed with Substance 4, Substance 21, Substance 25, and Substance 35.

4.3.3.5 False assumption about the initial cell density (Scenario 6, 7)

There are hints that in some cases the real initial cell density differed from the believed one. This error normally applies for cases in which the starting density was not directly measured but computed from the dilution of the stock-culture cell density. In the dilution procedure mistakes are possible, which is indicated by an unusual high or low growth rate from 0 to 24 h.

Figure 46 gives an example, in which in reality the initial density in all of the cultures was two-fold higher than assumed (Scenario 6). This type of error is also possible for a single treatment.

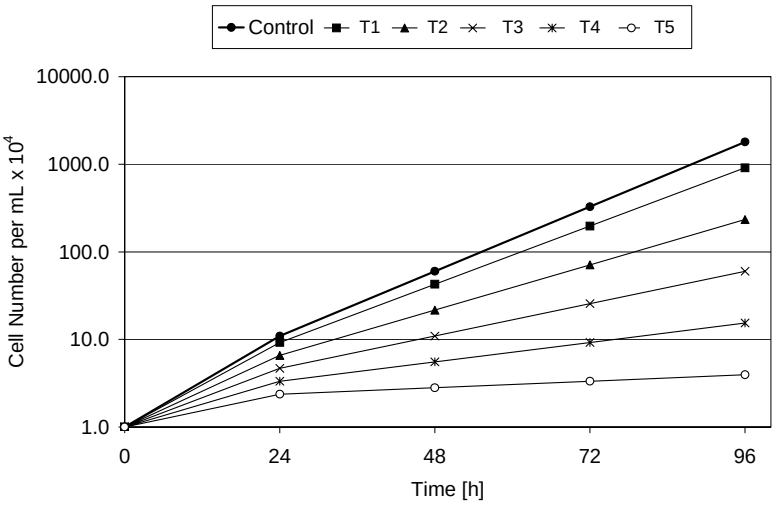


Figure 46: False assumption about initial cell density, which was two-fold higher than assumed (Scenario 6).

This sort of error might have been responsible for the growth pattern in Substance 12 (control), Substance 14, Substance 16 (highest concentration), and most probably in Substance 36.

The consequences of the inverse error - an assumption of a higher starting density than really true - are exemplified by Figure 47 (Scenario 7). In Scenario 7 the real initial density was two-fold lower than assumed. However, this sort of scenario cannot be distinguished from the lag-phase scenarios (Figure 44 and 45). Maybe, that also false-assumptions about the start density are among the above mentioned tests with lag-phases (e.g., Substance 30).

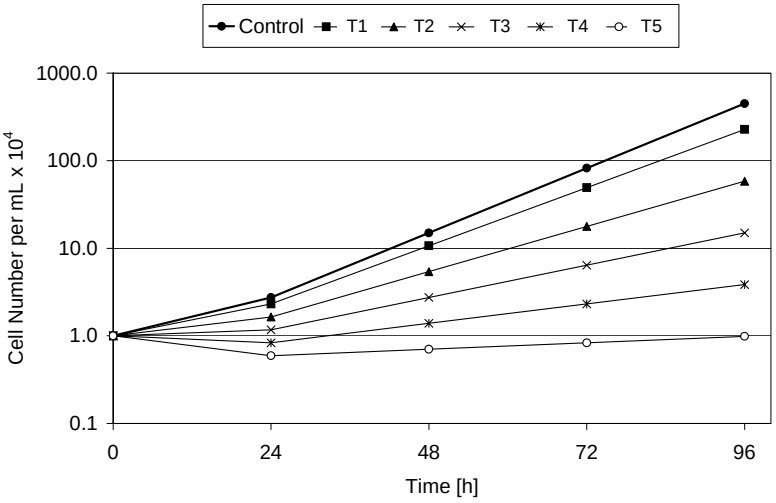


Figure 47: False assumption about the initial cell density, which was two-fold higher than assumed (Scenario 7).

4.3.4 Growth modification due to test-substance effects

The toxicity of a test substance varies, depending on the mode of toxic action, the uptake kinetics (bioaccumulation), stability of parent compound, generation of toxic metabolites, and effects on constituents of the culture medium, which could result in pH-changes and modified nutritional conditions. In these cases time-dependent alterations of the growth rate are possible and corresponding deviations from an exponential growth course can occur. Several examples of such type of growth modification will be given in the following.

4.3.4.1 Initial lag-phase in treated cultures (Scenario 8)

Lag-phases, occurring exclusively in the treated cultures, must be due to effects of the test substance. Theoretically, the substance can cause cell death in a part of the population, so that in the remaining more tolerant part of the population further growth can proceed, although

inhibited by the substance. Another effect could be an immediate reaction on the presence of the substance with a subsequent resumption of growth, since the concentration of the substance has fallen below a certain threshold or a less toxic metabolite has been formed. In other words, the effect has two phases: a stronger effect is replaced by a less strong effect.

This sort of growth pattern is shown in Figure 48 and examples for this are assumed in the tests with Substance 2, Substance 27 (second concentration), Substance 31 Test 2, Substance 33, and Substance 38.

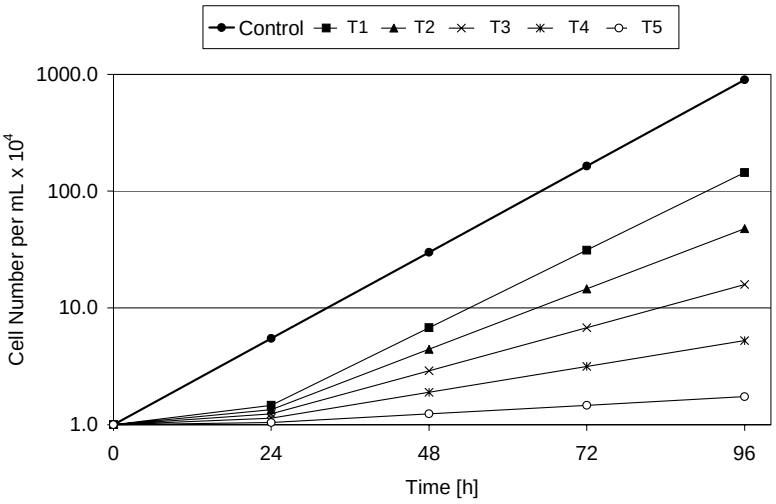


Figure 48: Growth delay restricted on the treated algae cultures during an initial lag-phase

4.3.4.2 Temporary toxicity during the initial test phase (Scenario 9)

If a substance is rapidly degraded to non-toxic products in the initial experimental period, the growth inhibition is only temporary and growth can subsequently proceed at the control rate, as is exemplified by Figure 49. Such sort of growth pattern could be realised in Substance 5, Substance 15 (?), Substance 23, Substance 31 Test 2, and Substance 38.

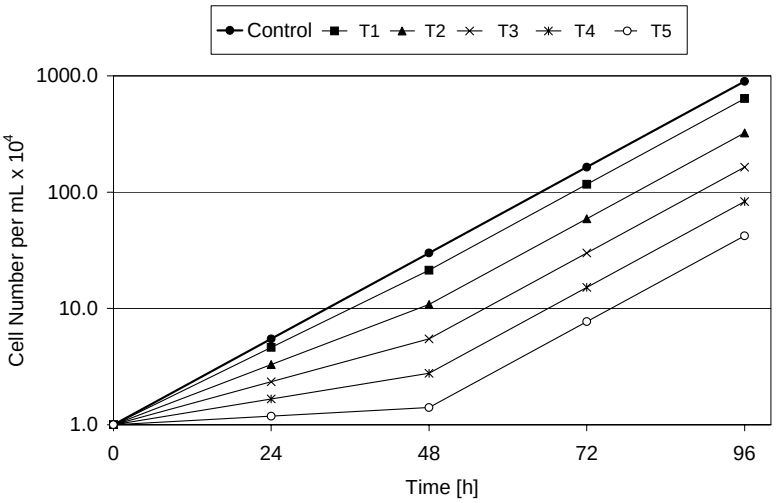


Figure 49: Temporary growth inhibition. Due to the instability of the test substance normal growth is resumed after 48 h.

4.3.4.3 Increasing toxicity during the test period (Scenario 10)

If a compound has to be accumulated, before it can act as a toxicant, the growth will be increasingly inhibited over time and generates a growth pattern as shown in Figure 50. Uptake kinetics of substances depend on their molecular characteristics (e.g., log K_{ow}, molecular size and structure). Similar curves arise from the production of (more) toxic metabolites from a non/less toxic parent compound during the test period.

Tests with Substance 7, Substance 8, Substance 26, and Substance 37 exhibited a respective growth pattern.

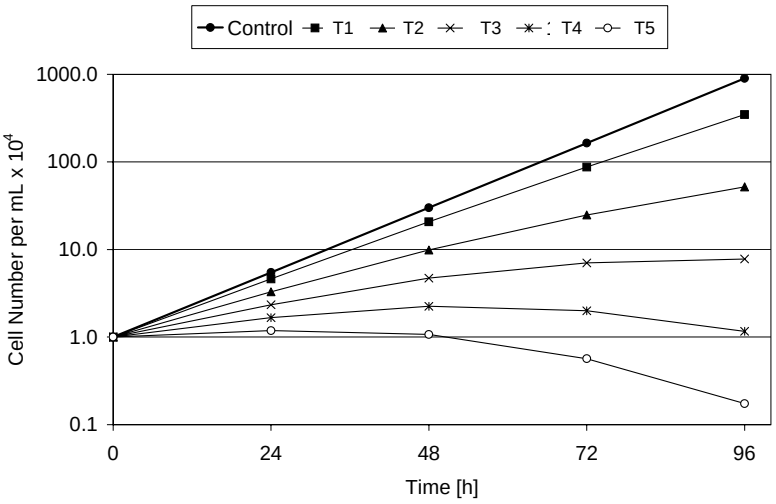


Figure 50: Increasing growth delay of algae cultures due to bioaccumulation of a toxic compound or to generation of a toxic metabolite during the test period.

4.3.4.4 Promoting effects at low concentrations (Scenario 11, 12)

The test substance can serve as metabolic stimulus for the promotion of growth, resulting in higher growth rates than in the control throughout the experiment (Figure 51, Scenario 11). Promoting effect can also be temporary, if a promoting compound is degraded (Figure 52, Scenario 12).

A Scenario-11-like growth pattern was found in Substance 2, Substance 8, Substance 9, Substance 11, Substance 19, Substance 20, Substance 21, Substance 30 Test 1, and Substance 38, whereas the Scenario 12 was not realised in the investigated tests.

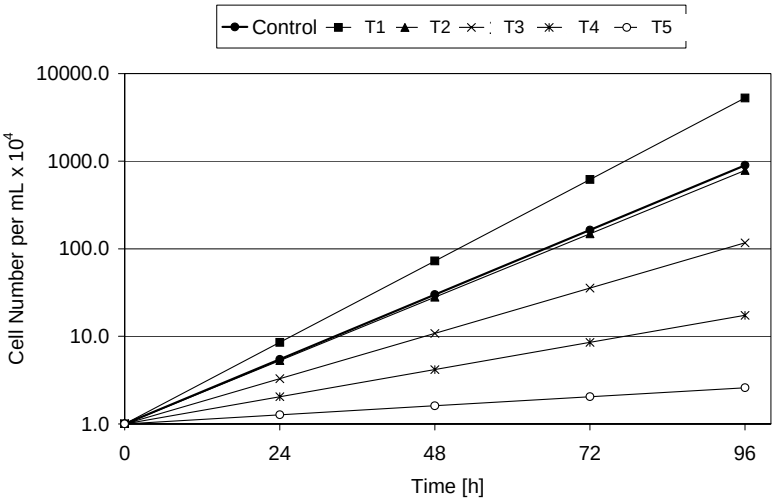


Figure 51: Growth promotion at low concentrations during the whole experimental time (Scenario 11).

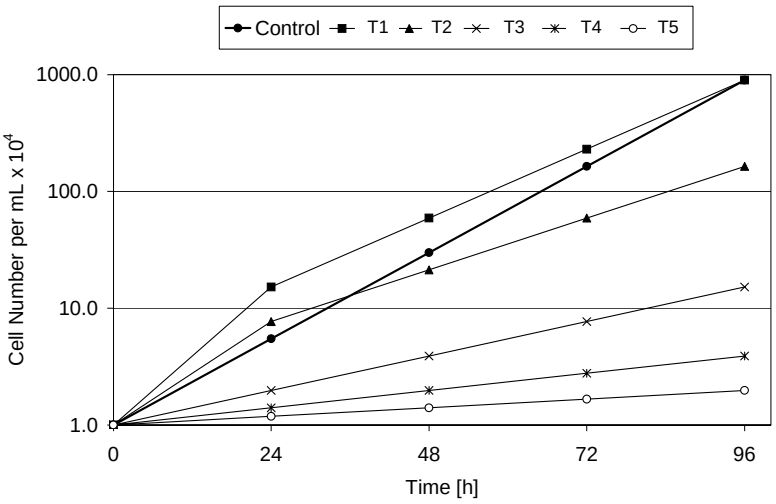


Figure 52: Temporary growth promotion at low test concentrations (Scenario 12).

4.4 Results on basic test parameters

This part of investigation was performed to extract as much information as possible from the data to get a complete picture about the basic characteristics of the response variables. For this it is comprised also of topics, which do not belong to the main subjects of the present study, but could be of importance in present and future discussions about statistical methods, validity criteria, etc.. Hence the detailed results are presented in Annex A (this volume). This section will briefly give some overview over the topics, results and conclusions.

- *Statistical distribution and variance homogeneity of response variables - prerequisites for parametric test methods*

  Generally, the proportion of normally distributed treatment and control data was higher than 80%, except for those of the first measuring interval, showing only about 70% normal distribution. The order in degree of normal distribution in controls was: Log (biomass integral) > Log (Cell count), Biomass integral > Growth rate > Cell count, Growth rate* (Figure A1 g). Marked effects of the test substances on the statistical distribution were not found. Overall, in “log (Cell count)” and “Growth rate” the highest homogeneity of variance was observed (Figure A2 g), followed by “log (Cell count)”, and “Growth rate”, all ranging between 70 and 80% homogeneity. In contrast, in “Cell count” and “Biomass integral”, homogeneity was about 50 to 74% and thus markedly lower.

  Summarising, the prerequisites “normal distribution” and “variance homogeneity”, as demanded by parametric statistical methods, can be seen as fulfilled to a great extent by both growth rates and “log (Cell count)”. However, it should be considered that the number of replicates, currently investigated in common AGITs, is relatively low and does often not allow the conduct of powerful statistical testing.

- *Prerequisites for dose/response curve modelling to derive an ECx*

  Dose/response[9] curve modelling includes finding an appropriate mathematical function, which describes the dose/response relationship and which can be used to calculate values for any ECx (so-called “point estimates”). Although curve fitting can be performed directly using the non-transformed response variable values, commonly the toxicant caused deviation is expressed as response relative to the control (Eq. 4). The relative expressions

[9] For the sake of simplicity, the common term “dose/response” is used, although for the aquatic environment “concentration/response” would be more adequate.

of the toxicant-caused modification of a response variable's value allow better comparison of dose/response curves from different substances or conditions. In many cases – in particular if the concentration range was appropriately chosen -, these relative responses follow a sigmoid curve with increasing log (concentration) and thus can be described by the normal sigmoid, the logistic and the Weibull function, for which linear transformations are existing.

In Annex A, it is demonstrated by the variances of generated %inhibition-data that variances are to a great extent non-homogeneous, due to the data transformation in %inhibition. This is a problem, since non-homogeneity of variances does not allow performing simple "normal" linear regression analyses. Therefore, the appropriate method is maximum likelihood (weighted) regression, as described by Finney (1971, 1978) and Weber (1980). Weighting functions for Probit-, Logit and Weibull-analyses of quantitative responses, as given here, are based on the Poisson distribution and are published, e.g., in Christensen (1984). Thus the AGIT data sets under consideration here allow to compute the ECx by means of a Probit, Logit and Weibull maximum-likelihood regression analysis, provided these functions can be appropriately fitted to the data. In the present study, probit analyses were performed throughout, which in the overwhelming number of cases was suitable.

- *The behaviour of test parameters used or discussed as validity criterion*

  Figure A5 presents control growth rates, as observed for the various substances at different observation times, gives minimum average growth rates, as prescribed by various guidelines, and shows the belt where the mean control growth rate is seen as optimal (DIN 1993). Less than one third of the control test cultures showed optimal average growth rates. Two tests did not fulfil the OECD/ISO/DIN validity criterion, but all of the tests met the US-EPA criterion. According to the theoretical considerations given in Section 2.6 and simulated results (shown below), the growth rate level is predicted to affect the E_rC_{50}/ E_bC_{50} ratio.

  The coefficient of variation (CV) is being discussed as an additional validity criterion of the AGIT (Nusch, pers. comm.). In the currently used response variables, after 72 h the CVs were highest in „Cell count" (16.24%), followed by „Biomass integral" (15.71%) and „Growth rate" (8.32%). The latter represents the average rate over time and thus has lower CVs than the section by section growth rate ("Growth rate*") in which the values were similar to those in "Cell count".

4.5 Comparison of toxicity parameters

Also this part of investigation did not belong to the main subjects of the present study, but was conducted for the same reasons as above. The comparisons were made for the 72 h test period. Also here the detailed results are presented in Annex A, whereas in the following a brief summary of topics, results and conclusions is presented.

- *Submitted results vs. those from the present study*

  In a substantial number of tests the EC_{50} from the present study differed markedly from those reported to the German Federal Environmental Agency, obviously due to different methods used for the EC_{50} computation. The conclusion from this comparison thus is that the statistical methods need more standardisation and harmonisation.

- *Statistical test, data transformation and the NOEC*

  It was examined whether the log-transformation of "Cell count", which appears as the only way to use "Cell count" with parametric tests, leads to less sensitive (higher) NOECs or not. The log-transformation might lead to less sensitive NOECs as for example is sometimes the case in "Growth rate". In addition, the NOECs from transformed data was compared with those from the Bonferroni-U test – the non-parametric alternative test procedure (Table A2).

  The conclusion was that, if necessary, the transformation should be used rather than the non-parametric test alternative which leads to less sensitive NOECs in a substantial number of cases. As was expected from the different power of the considered statistical tests (Williams > Dunnett > Bonferroni-U), in many cases the NOECs was lowest and sometimes even more reasonable, if the most powerful Williams test was applied (Table A3 and A4). The degree of normal distribution and variance homogeneity in the data appears to be high, so that parametric tests normally can (and should) be performed. From these the Williams test should be preferred due to it higher statistical power and its ability to smooth the dose response relationship, so that more reasonable NOECs will be obtained.

- *Comparison of EC_{10}, EC_{20} and EC_{50} with the NOEC*

  In order to provide background information on which of the ECx might be appropriate for replacement of the NOEC the ratio ECx/NOEC was investigated for the response variables (Figure A7). In "Cell count" and "Biomass integral" the NOEC corresponded nearly the EC_{20}, in the section by section growth rate (Growth rate*) some value between EC_{20} and EC_{10} and in the "Growth rate" the EC_{10}.

  For the final conclusions one has to consider, that the NOEC is a rather conservative estimate of the NEC at higher variances and low replication, since significant results (LOECs) are obtained at concentration exerting already a substantial effect. Hence the NOEC should not be used for the calibration of any ECx. This is especially true at higher variances and low replication. Therefore, it would be no improvement if in the "Cell count" and "Biomass integral" the NOEC would be replaced by the EC_{20}. In contrast, in the growth rates with lower variances the NOEC might be replaced by the EC_{10}. If a decision like that would be made, only those tests should be accepted in which a clear dose/response relationship is observed and thus a function can be fitted from which an ECx (x = small) can be derived.

4.6 The ratio between the ECx in various response variables

4.6.1 The ratio E_rC_{50}/E_bC_{50}

The ratio between the biomass parameter (Cell count, Biomass integral) and the growth rate has been already discussed with the report of the literature findings (Section 2.6). Predictions about the ratio E_rC_{50}/E_bC_{50} were derived from the mathematical relationship between the dose/response functions under certain assumptions. Indeed, these predictions were often confirmed by experimental results (e.g., Dorgerloh 1997). However, in a substantial number of cases extreme high or low ratios of E_rC_{50}/E_bC_{50} were to be observed. One of the main purposes of this study is to find out reasons for these obviously deviating results. Among the reasons could be properties of the substance, experimental problems (inconstancies, nutrient

limitation) and statistical artefacts. Again, comparisons were made only for the 72 h test period.

Figure 53 shows the ratio of E_rC_{50}/E_bC_{50}, ordered by magnitude, for the various substances. Those results, in which the EC_{50} was in the range of the 0.1-fold of the lowest and the 1.5-fold of the highest test concentration, are indicated by broad bars. Obviously, with three exceptions (Substance 36, two tests with Hymexazol) the ratios were in the predicted range of 1 to 10. The majority of higher ratios were found in cases where the EC_{50} was far outside the tested concentration range, strongly suggesting that statistical problems in appropriately modelling the dose/response curve were the reason. Only in 4 of 14 cases, ratios between 10 and about 200 were obtained, in which the EC_{50} was in the range of tested concentrations.

Figure 54 compares the ratio of E_rC_{50}/E_bC_{50} with the ratio of E_rC_{50}/E_cC_{50}. As was to be expected, with 17 greater E_rC_{50}/E_cC_{50} ratios and 18 lower ones the overall picture was quite similar, although in single cases markedly different ratios were observed. Hence it will be sufficient to focus the following considerations on the ratio of E_rC_{50}/E_bC_{50} alone.

4.6.2 Reasons for extreme ratios of E_rC_{50}/E_bC_{50}

4.6.2.1 Ratios of $E_rC_{50}/E_bC_{50} < 1$

According to the predictions by Eq. 5 (Section 2.6; Nyholm 1985) ratios of $E_rC_{50}/E_bC_{50} < 1$ are to be expected shortly after the start of a test within the first 24 h. Nonetheless, ratios < 1 were to be observed after 72 h, too. This applied for Substance 20 (ratio 0.71) and Substance 21 (ratio 0.63). A closer examination of these test revealed that in both tests there was a growth promotion up to the second highest concentration, followed by 100% inhibition in the highest concentration, so that there were no intermediate inhibitions and the dose/response relationships was extremely steep. These conditions probably were responsible for the extreme low ratios. They are seen as inappropriately chosen by the experimenter and such sort of test should not be accepted as valid.

4.6.2.2 Ratios of $E_rC_{50}/E_bC_{50} > 1$

To check whether statistical problems could be responsible for the extreme ratios of E_rC_{50}/E_bC_{50}, the following hypothesis was investigated:

Large ratios of E_rC_{50}/EbC_{50} are caused by the fact that both response variables are affected differently by the inhibition of algal growth. In other words, values of %inhibition in “Growth rate” and “Biomass integral” are different and thus located in different areas of the does/response curve (further called “dissimilar distribution”). Low differences in the “Growth rate” cause relatively high differences in the “Biomass integral”. Therefore, at moderate inhibitions in “Growth rate” higher %inhibitions in “Biomass integral” (e.g., > 50%) are more probable so that the EC_{50} is not located in the centre of the measured values and often has to be extrapolated. The inverse is true for the average growth rate, which at lower inhibitions exhibits %inhibitions < 50%. Flat dose/response curves which extend over several orders of magnitudes enhance this effect, which is in principle also predicted by Eq. 5 (Section 2.6; Nyholm 1985). This shall be demonstrated using some substances causing a high ratio of E_rC_{50} / E_bC_{50}.

To quantify the dissimilar distribution of %inhibitions, the difference between the number of %inhibitions <50% and >50% in both response variables was used as a dissimilarity measure (further called „degree of dissimilar distribution“ (ddd)).

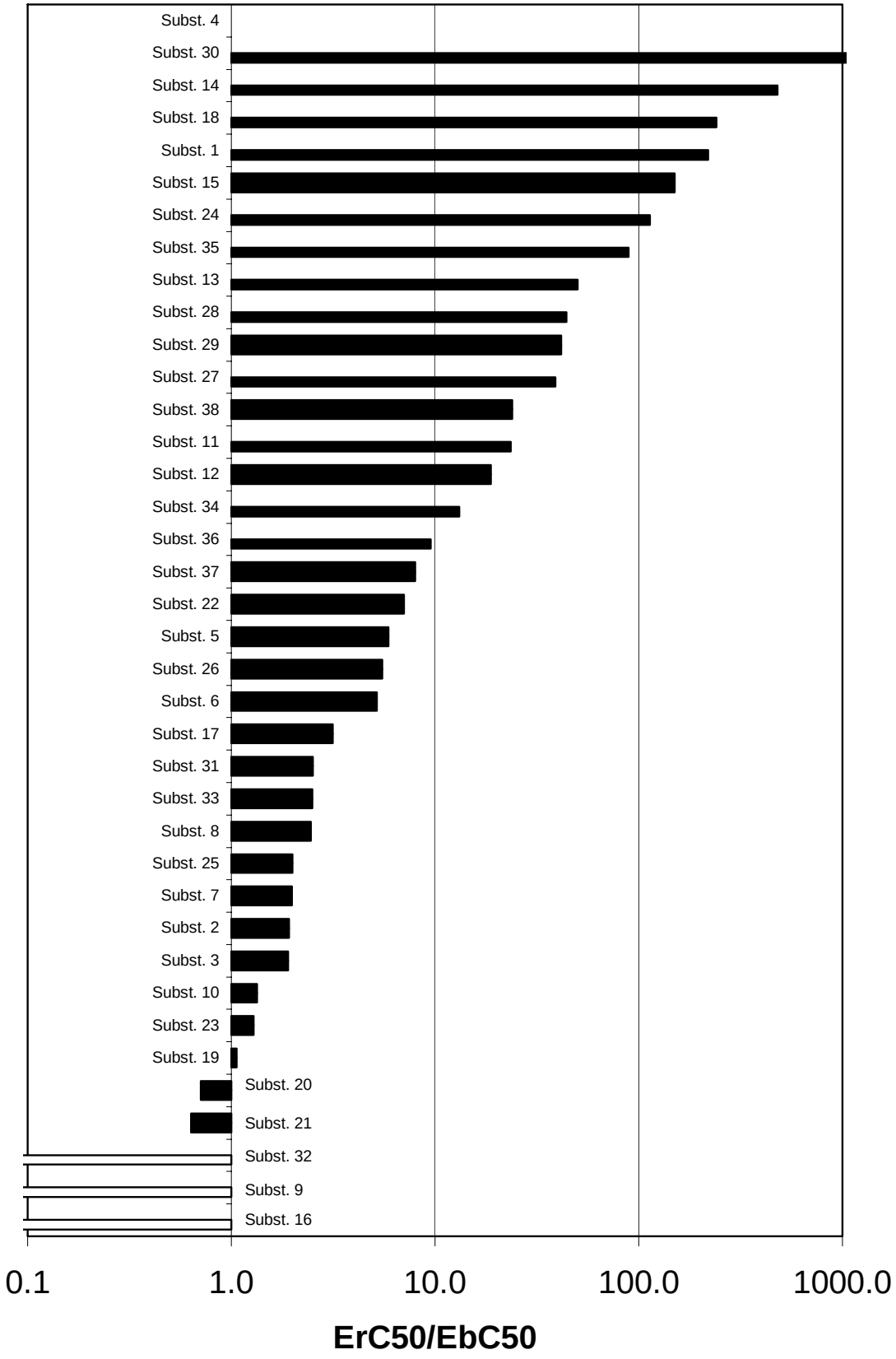


Figure 53: The ratio E_rC_{50}/E_bC_{50} as observed at 0 – 72 h for the various test substances. Broad bars indicate that the EC_{50} was in the range between the 0.1-fold of the lowest and the 1.5 fold of the highest test concentrations. In small bars the EC_{50} was found outside this range. White bars indicate abnormal ratios < 1 (probably statistical artefacts).

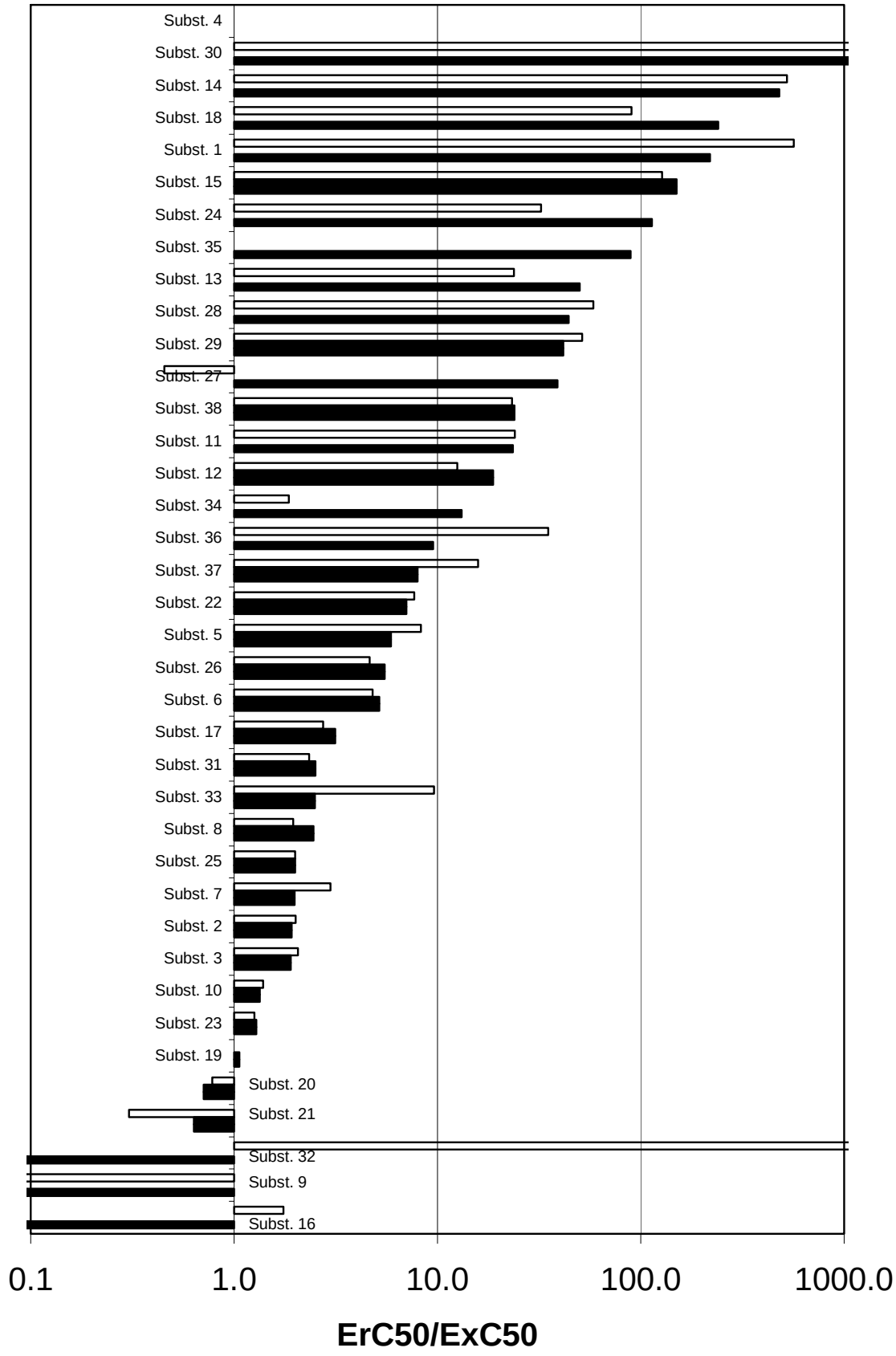


Figure 54: Comparison of the ratio E_rC_{50}/E_bC_{50} (black bars) with the ratio E_rC_{50}/E_cC_{50} (white bars) as observed at 0 – 72 h for the various test substances. Broad black bars indicate that the EC_{50} was in the range between the 0.1-fold of the lowest and the 1.5 fold of the highest test concentrations. In small bars the EC_{50} was found outside this range.

The examples of Table 5 confirm the above hypothesis. The inhibitions proved to be distributed very dissimilarly over the percentage range (mean degree of ddd for these cases = 4.4). While in "Growth rate" the inhibitions are located predominantly in the lower percentage range, in "Biomass integral" these were found mainly in the upper range. In the substances considered as yet the dose/response curves on average extended over 2.74 orders of magnitude.

According to the above hypothesis there should also be substances with a lower E_rC_{50} / E_bC_{50} ratio (e.g., 1 - 2) and with inhibitions, distributed more or less similarly over the percentage range. Examples are given in Table 6.

Table 5: Examples for a high degree of dissimilarity and large ratios of E_rC_{50} / E_bC_{50}; for explanation see text

a) Substance 15; ratio 150

Response variable	**Range [mg/L] (orders of magnitudes)**	**Inhibition range**	**# < 50%**	**# > 50%**
	0.158 - 500 (3.5)			
Biomass Integral		- 0.5 – 91.2 %	2	6
Growth rate		- 7.0 - 63.0 %	7	1
Degree of dissimilar distribution (ddd)			5	5

b) Substance 18 1; ratio 240

Response variable	**Range [mg/L] (orders of magnitudes)**	**Inhibition range**	**# < 50%**	**# > 50%**
	0.013 - 0.8 (1.8)			
Biomass integral		9.1 - 70.6 %	2	5
Growth rate		1.5 - 28.9 %	7	0
Degree of dissimilar distribution (ddd)			5	5

c) Substance 35; ratio 89

Response variable	**Range [mg/L] (orders of magnitudes)**	**Inhibition range**	**# < 50%**	**# > 50%**
	0.47 – 190 (2.6)			
Biomass integral		22.8 - 87.5 %	2	4
Growth rate		7.7 - 45.5 %	6	0
Degree of dissimilar distribution (ddd)			4	4

Table 5 (continued)

d) Substance 13; ratio 50

Response variable	Range [mg/L] (orders of magnitudes)	Inhibition range	# < 50%	# > 50%
	0.1 - 220 (2.3)			
Biomass integral		17.7 - 97.0 %	2	6
Growth rate		- 1.4 - 30.7 %	7	1
Degree of dissimilar distribution (ddd)			5	5

e) Substance 29; ratio 42

Response variable	Range [mg/L] (orders of magnitudes)	Inhibition range	# < 50%	# > 50%
	0.36 - 1200 (3.5)			
Biomass integral		10.2 - 93.0 %	3	5
Growth rate		2.2 - 63.9 %	6	2
Degree of dissimilar distribution (ddd)			3	3

Table 6: Examples for a low degree of dissimilarity and small ratios of E_rC_{50} / E_bC_{50}; for explanation see text

a) Substance 23; ratio 1.29

Response variable	Range [mg/L] (orders of magnitudes)	Inhibition range	# < 50%	# > 50%
	0.39 - 50 (2.1)			
Biomass integral		5.0 - 107.6 %	4	4
Growth rate		2.1 - 100 %	4	4
Degree of dissimilar distribution (ddd)			0	0

Table 6 (continued)

b) Substance 19 ; ratio 1.06

Response variable	Range [mg/L] (orders of magnitudes)	Inhibition range	# < 50%	# > 50%
	6.25 - 100 (1.2)			
Biomass integral		-22.1 - 100.1 %	3	2
Growth rate		- 3.2 - 100.0 %	3	2
Degree of dissimilar distribution			0	0

c) Substance 10; ratio 1.34

Response variable	Range [mg/L] (orders of magnitudes)	Inhibition range	# < 50%	# > 50%
	0.0625 - 1.0 (1.2)			
Biomass integral		- 1.8 - 72.4 %	4	1
Growth rate		- 0.7 - 50.2 %	4	1
Degree of dissimilar distribution			0	0

Also these examples are in line with the above hypothesis. The inhibition in “Biomass integral” and “Growth rate” were distributed similarly and the calculated EC_{50}´s differed only negligibly. The degree of dissimilar distribution (ddd) is zero for these substances. Furthermore, the dose/response range extended only over 1.5 orders of magnitudes.

In order to show the general validity of the hypothesis, in Figure 55 the factor E_rC_{50} / E_bC_{50} is plotted over the degree of dissimilar distribution for all substances, in with sufficient data were available.

Extremely large ratios of E_rC_{50}/E_bC_{50} could not be attributed to special properties of the substance. This is supported by tests in which the same substances were tested several times (Substance 18 - 21, Substance 30-31). It could be shown that large ratios of E_rC_{50}/E_bC_{50} are mainly caused by the fact that the concentration range, chosen for a test substance, is not equally suitable for “Biomass integral” and “Growth rate” and more so, if the dose/response curve is flat and extends over a large concentration range. In these cases, it is practically impossible to select a concentration range, being appropriate for both response variables.

Using simulated AGITs, it will be shown below that the major reason for the relation of Figure 53 is the variability in the inhibition data, which impairs finding the correct parameter values of the “true” dose/response function.

The lesson to be learned from these findings is that there is an urgent need to focus on only one of the response variable for the endpoint “algal growth”, so that for this variable the experimental conditions, e.g., the concentration range, can be optimised.

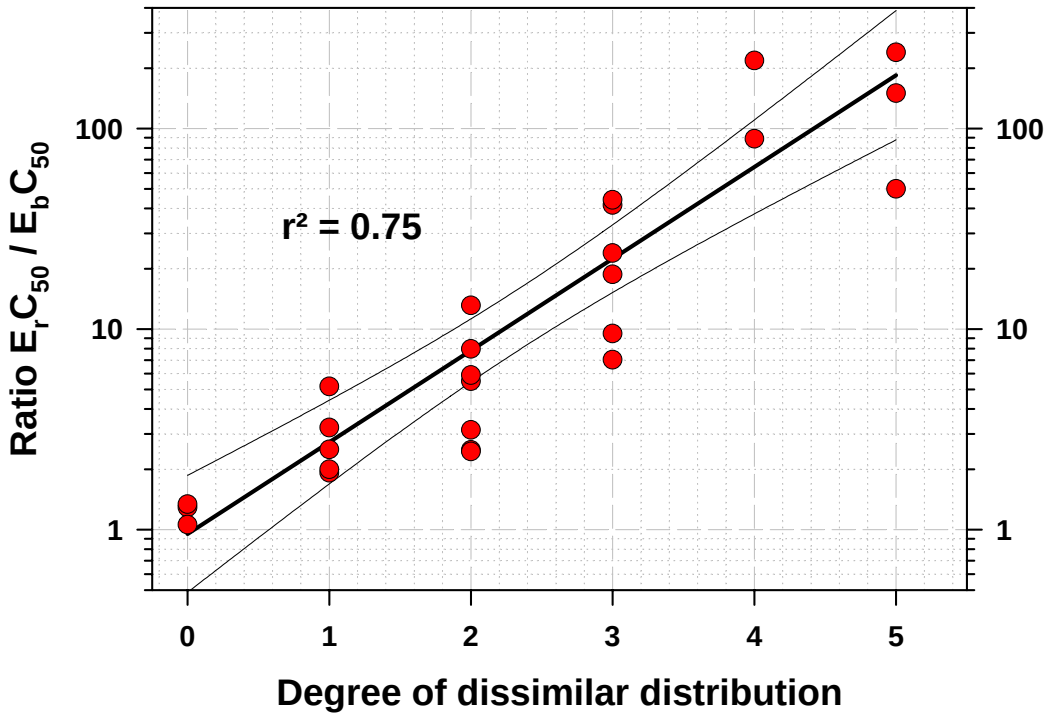


Figure 55: Relationship between the degree of dissimilar distribution of inhibitions and the ratio E_rC_{50}/E_bC_{50}; shown are the data points and the linear regression line with 95%-confidence belt

4.6.3 Comparison of EC_{50} ratios of various response variables

One of the reasons for the lower sensitivity of "Growth rate" certainly is the fact that this response variable averages over the growth curves, whereas "Biomass integral" sums up all deviations in the growth (thus also the variability). In this section, other ratios of the EC_{50} will be examined. In addition, since the NOEC could be replaced by some ECx (x << 50), also the ratio E_rC_{10}/E_bC_{10} and E_rC_{20}/E_bC_{20} will be dealt with.

4.6.3.1 Ratio between the section by section growth rate and biomass integral ($E_{r*}C_{50}/E_bC_{50}$)

In Figure 56, the ratio $E_{r*}C_{50}/E_bC_{50}$ is compared with the ratio E_rC_{50}/E_bC_{50} (from Figure 53). In 67% of cases "Growth rate*" was more sensitive than "Growth rate", but was always less sensitive than "Biomass integral".

4.6.3.2 Ratio between the most sensitive growth rate and biomass integral ($E_{r\text{-}min}C_{50}/E_bC_{50}$)

The average growth rates smooth deviations in the course of growth. Especially in the control, if the growth slows down due to some limiting factor, the average growth rate becomes slower, which makes the growth rate less sensitive in effect monitoring. It can be discussed, whether one should use the growth rate when it is most sensitive. This for example can be

during a time period, before limitation started. To examine this, the growth rate was selected from 0-24 h, 0-48 h, and 0-72 h, which showed the smallest EC_{50} ($E_{r\text{-}min}C_{50}$).

Figure 57 demonstrates that the ratio $E_{r\text{-}min}C_{50}/E_bC_{50}$ was in 27 cases smaller than the ratio E_rC_{50}/E_bC_{50} (from Figure 53). In 10 of the cases the ratios were equal. Hence, using the most sensitive value of "Growth rate", reduces the difference in the results between "Growth rate" and "Biomass integral".

4.5.3.3 Ratio between the most sensitive section by section growth rate and biomass integral ($E_{r*\text{-}min}C_{50}/E_bC_{50}$)

Figure 58 demonstrates that the ratio $E_{r*\text{-}min}C_{50}/E_bC_{50}$ was in all cases smaller than the ratio E_rC_{50}/E_bC_{50} (from Figure 53). In the overwhelming majority of cases this ratios exhibited values between 1 and 10. Hence, using the most sensitive value of "Growth rate*", reduces the difference in the results between "Growth rate" and "Biomass integral" to the greatest extent.

4.6.4 Comparison EC_{10}, EC_{20} and EC_{50} ratios of various response variables

Figure 59 compares ratios of various EC-values over time. In so doing, from all tested substances the median ratio was computed each, thus excluding the effect of the extreme, non-reasonable ratios.

Independently of experimental time, from the EC_{50} down to the EC_{10}, the EC ratio of "Growth rate/Biomass Integral" decreased on average from 5.9 to 3.4, that of "Growth rate*/Biomass Integral" from 3.9 to 3.4, that of "Growth rate/Cell count" from 4.9 to 3.0 and that of "Growth rate*/Cell count" from 2.6 to 2.3. The EC ratio "Growth rate/Growth rate*" varied around 1. On the other hand, the ratios on average increased with increasing time.

These observations are in line with the predictions of Nyholm (1985; Section 2.6). If the NOEC would be replaced by an EC_{10} or EC_{20}, lower ratios in the ECx between the biomass and growth rate parameters are to be expected.

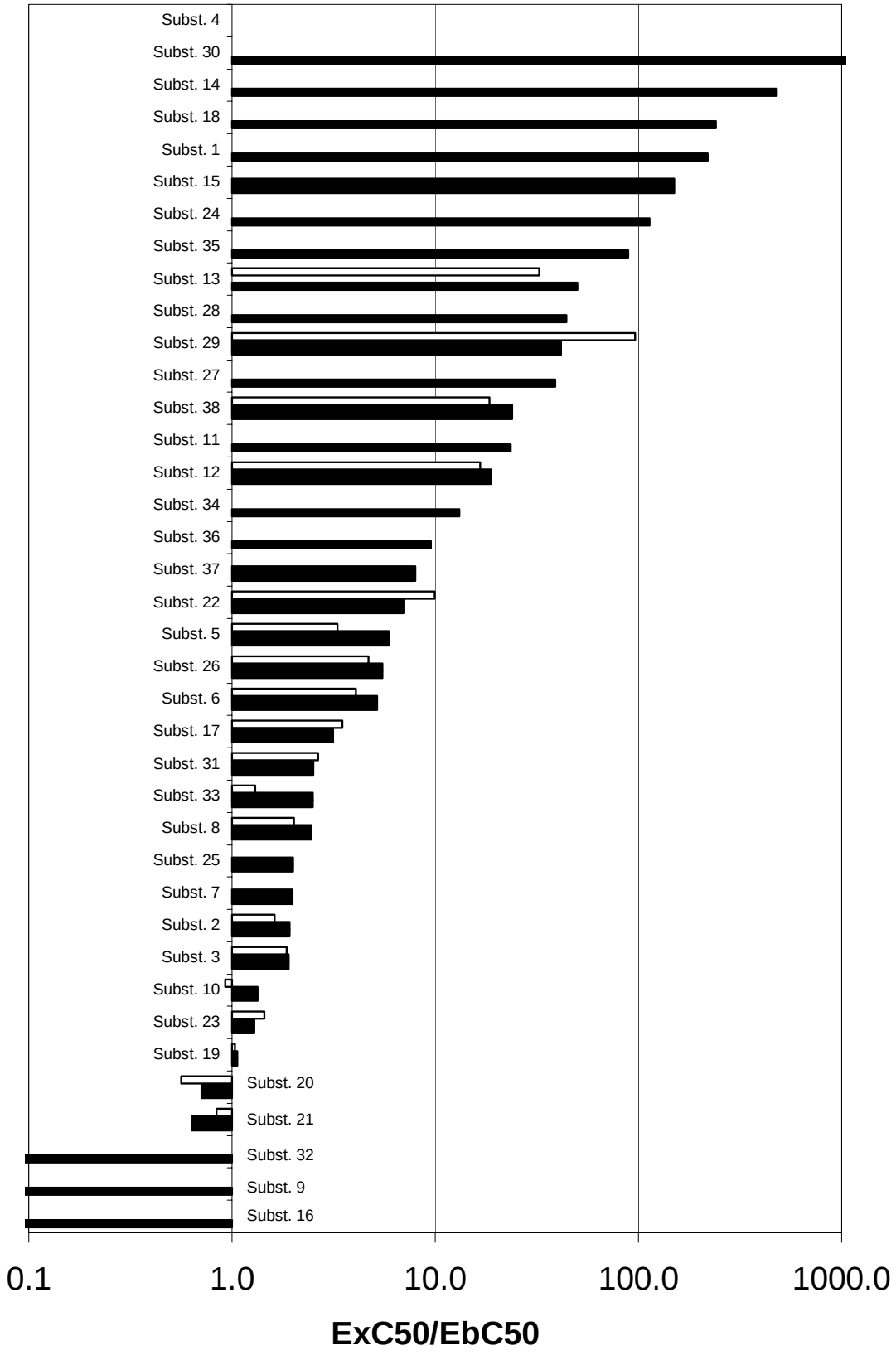


Figure 56: Comparison of the ratio E_rC_{50}/E_bC_{50} (black bars) with $E_{r*}C_{50}/E_bC_{50}$ (white bars) as observed at 0 – 72 h for the various test substances (r* = section by section growth rate 48-72 h). Broad bars indicate that the EC_{50}s were in the range between the 0.1-fold of the lowest and the 1.5 fold of the highest test concentrations. In small bars the EC_{50}s were found outside this range. Missing white bars indicate that in these cases the $E_{r*}C_{50}$ could not be determined for this time period.

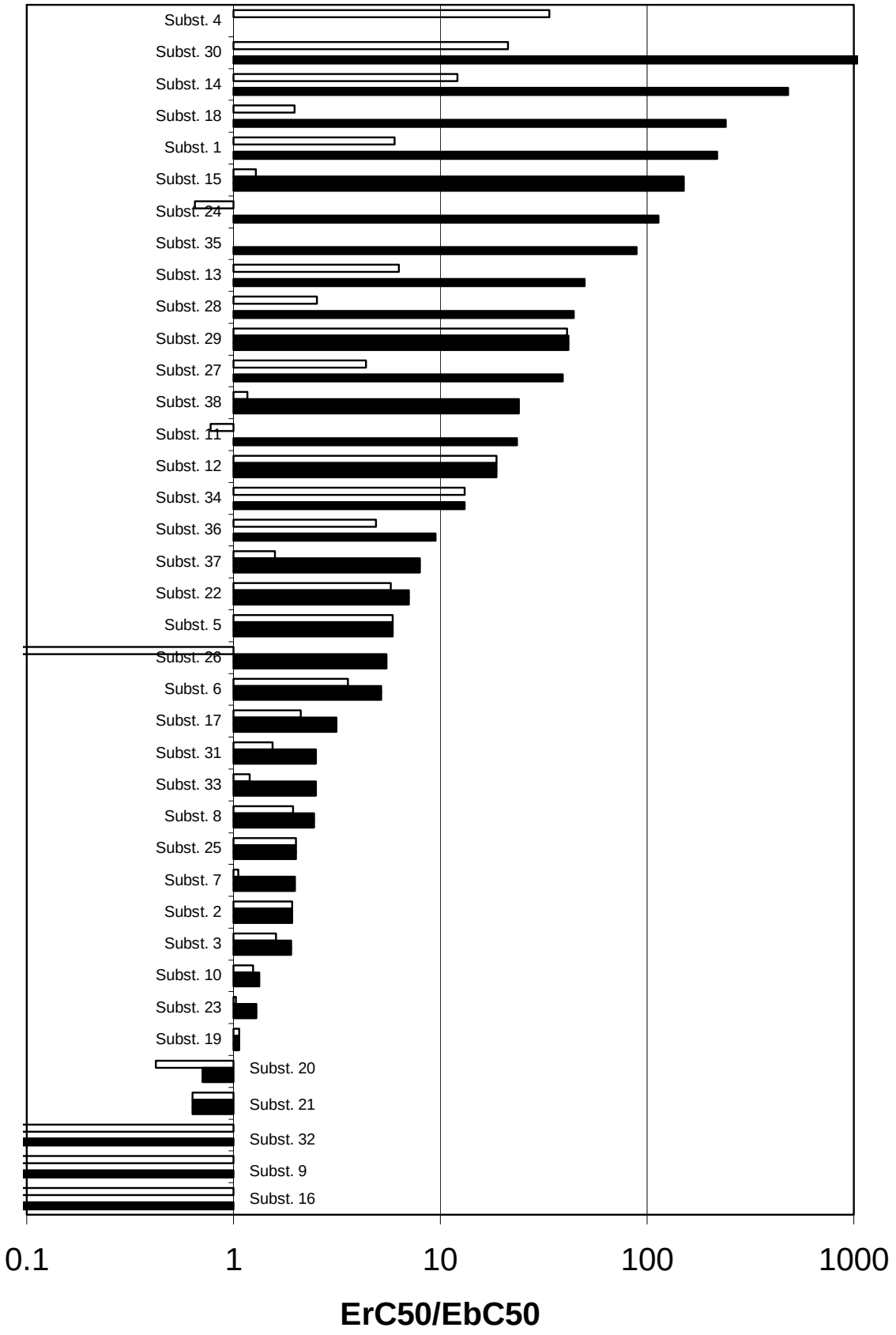


Figure 57: Comparison of the ratio E_rC_{50}/E_bC_{50}, where r was averaged from 0-72 h (black bars), with $E_{r\text{-}min}C_{50}/E_bC_{50}$, the minimum of r (0-24, 0-48, 0-72h; white bars) as observed for the various test substances

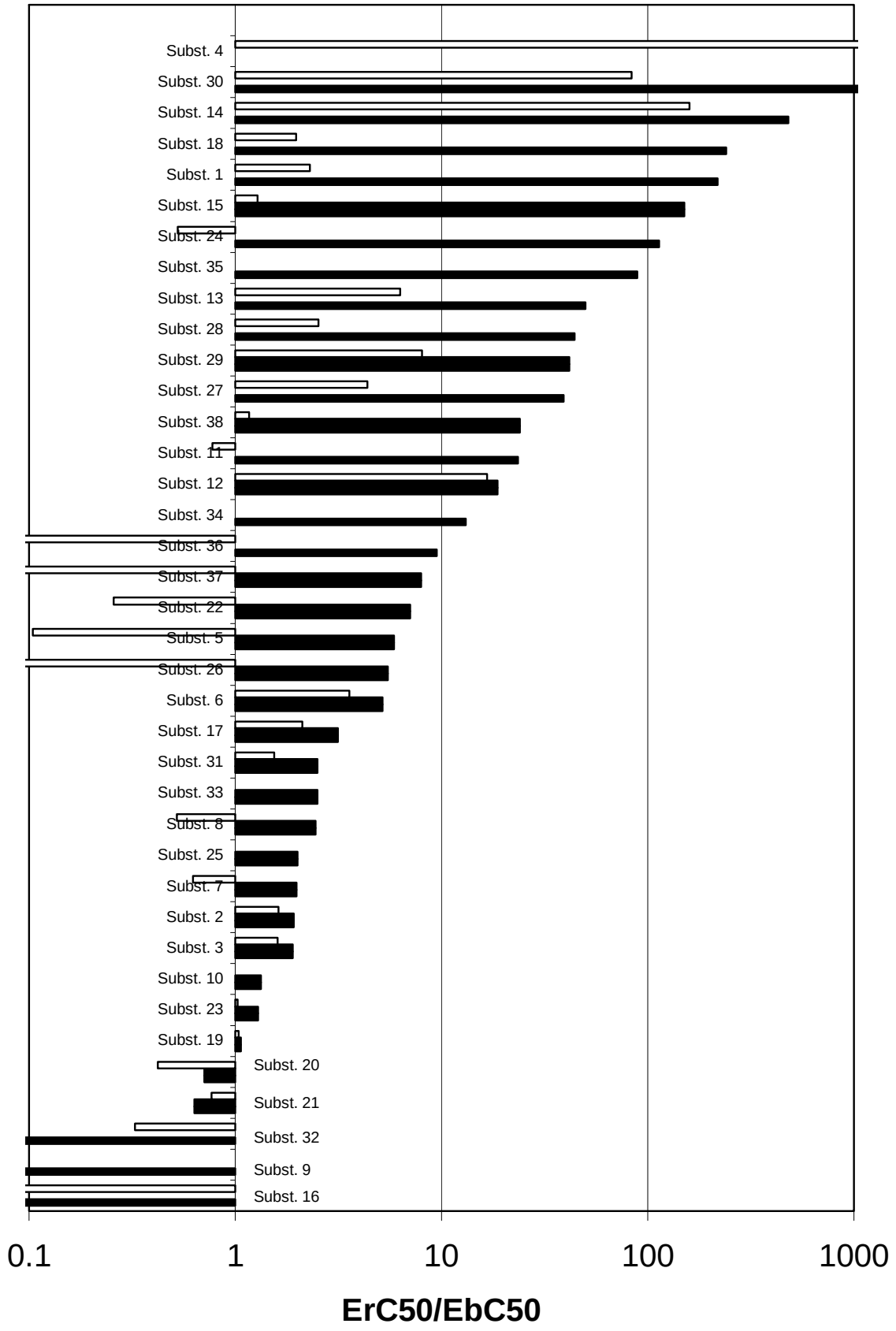


Figure 58: Comparison of the ratio E_rC_{50}/E_bC_{50}, where r was averaged from 0-72 h (black bars), with the $E_{r^*\text{-}min}C_{50}/EbC_{50}$, the minimum of the section by section Growth rate r* (0-24, 0-48, 0-72h; white bars) as observed for the various test substances

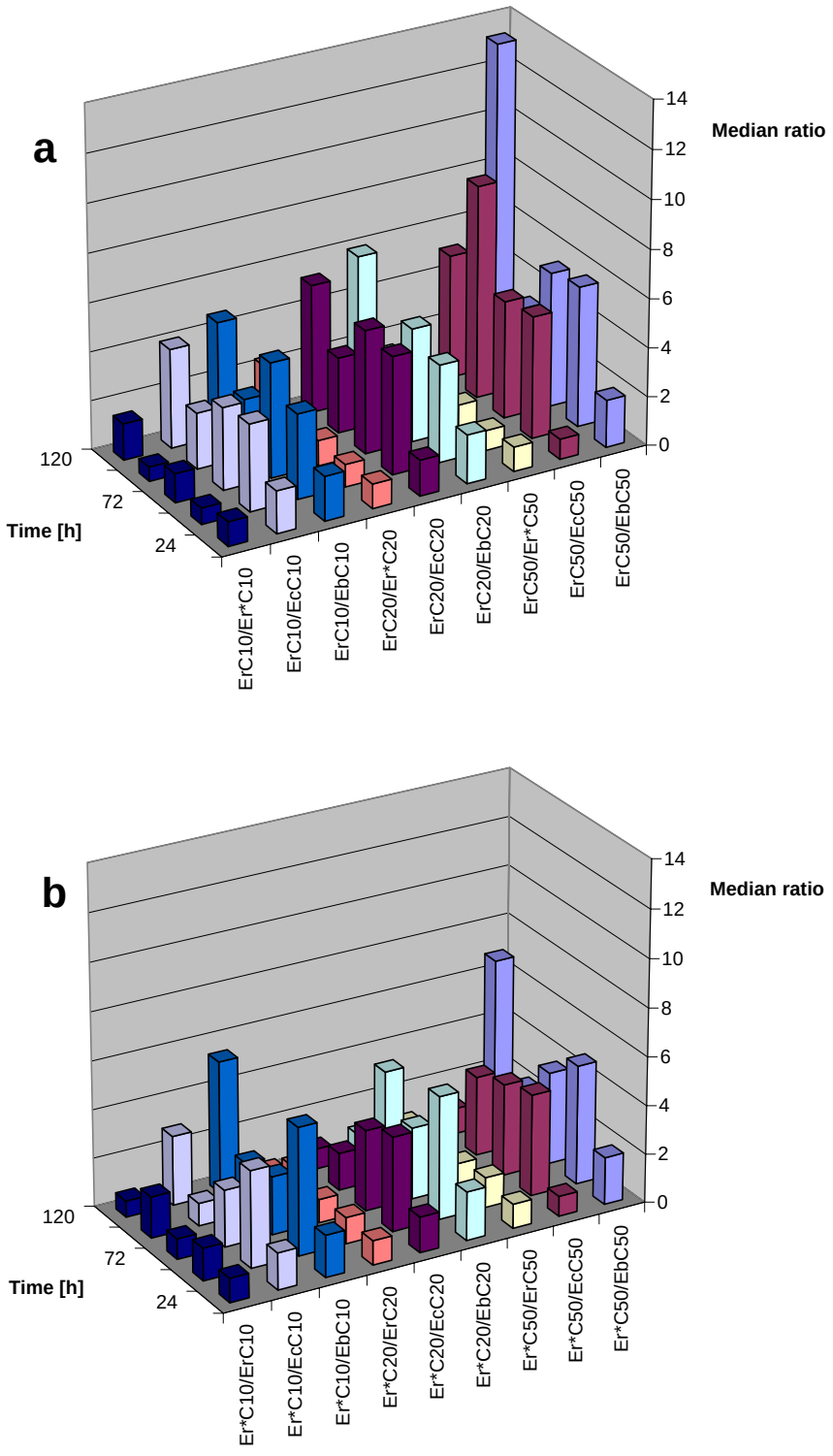


Figure 59: Comparison of the median ratios of $E_rCx/EyCx$ (a) and of $E_{r*}Cx/EyCx$ (b), where x = 10, 20, 50 and y = b, c, r*(r) (a(b)); for explanation see text

4.7 Concluding remarks about the results with submitted test data

From the analysis of the growth curves, performed as yet, and the evaluation of the basic characteristic of the response variables and toxicity parameters the following conclusions can be drawn:

- The basic growth pattern was exponential in many cases – at least for some period of time. Deviations are probably caused by substance characteristics and experimental shortcomings, which will be further investigated in the following Chapter.
- The observed growth pattern could be attributed to some characteristic scenarios. Obviously a substantial number of growth patterns and low growth rates in some control cultures are due to poor experimental conditions.
- For use with parametric statistical tests, both growth rate variants and "Log(Cell count)" proved to be appropriate rather than "Biomass Integral". Also with dose/response modelling, a low variance as observed with "Growth rate" is beneficial in finding the appropriate function and in computation of confidence limits.
- With statistically testing of "Cell count", the log-transformation together with a parametric test should be performed rather than a non-parametric test.
- For the determination of a NOEC Williams test procedure appeared as more appropriate than Dunnett's test or the non-parametric Bonferroni-U test.
- If the response-variable values are transformed into relative values (e.g., %inhibition), only the weighted maximum-likelihood regression analysis with probits, logits or the Weibull-transforms should be performed or a non-linear regression procedure. The suitability and performance of these methods, allowing the choice of the most suitable method, was not investigated further by the present study. This would be worth to be investigated further by an additional study.
- If the NOEC shall be replaced by some ECx, the EC_{10} appears more appropriate than another ECx, where $x > 10$.
- Extreme EC_{50}-ratios between the growth rate and the biomass parameter are clearly due to an inappropriate choice of the concentration range. This results in an inappropriate extrapolation of the EC_{50} and strongly biased estimations of its value.
- The ECx-ratio between the growth rate and the biomass parameter is lower, if the most sensitive ECx of the growth rate from the investigated time periods is used and/or if the section by section growth rate is inserted. The EC_{10}-ratios are lower then the EC_{50}-ratios.

The overall conclusion is that to a great extent the extreme results in EC_{50}-ratios were due to experimental shortcomings. Therefore it should be urgently recommended that proper experimental conditions are adjusted, the endpoint "growth" should be represented by one response variable, thus ensuring the concentration range to be chosen appropriately, and that the statistical evaluation should be standardised and monitored by the competent authorities.

In the following chapter, a generalised consideration and evaluation of simulated AGITs will aid the selection of the most appropriate response variable, which then should be exclusively chosen for the determination of any toxicity parameter.

5. Effects of growth course on the response variables - theoretical example scenario analysis

5.1 Introduction

In this chapter, theoretical test scenarios and simulated AGITs will be used in order to draw some general conclusions about the behaviour of the response variables under different conditions or scenarios. Among the benefits of such type of approach are: (1) The growth pattern can be set (e.g., strictly exponential), (2) the toxic action of a substance can be definitely set according to a known dose/response relationship, and (3) interference factors can be superimposed in a manner that inhibitions or promotions can be modelled (e.g., nutrient and carbon dioxide limitation, temperature drops, degradation of the parent compound, different variability). In addition, also the slope of growth curve, the slope of the dose/response relationship and the variation of the data points (measured cell densities) can be modified in definite known quantities so that the consequences of these settings for the various response variables can be systematically studied.

As with any population growth, the simulation of the algal population growth requires a staring population size and a growth rate, r, describing the change of population size over time. The characteristic of this change may be different; linear, exponential or other. However, in micro-organisms and many small invertebrates - the so-called r-selected organisms - exponential growth is typical, since they are prepared to colonise new habitats and are often growing far below the habitat's carrying capacity under non-limiting conditions. Undoubtedly, the exponential growth pattern is characteristic also in the algae species used in toxicity tests. This was frequently demonstrated by exponential growth in the controls and was found also with the submitted tests of the present study, where exponential growth was observed both in the controls and in the treatments - at least lasting for some period of time. Deviations from the exponential pattern could be interpreted as probably due to interference factors, disturbing the maintenance of a constant growth rate. The interference may be caused for example by inconstant experimental conditions, nutrient limitation, and the toxicant itself, undergoing some change (e.g., bioaccumulation, degradation) during the experimental time. These interference by various factors do not justify to have general doubts about the underlying basic exponential growth pattern in the considered algae. Therefore, the exponential growth pattern was chosen as the underlying mode of growth for all of the simulations, presented below, and interference functions were superimposed on this pattern to cope for the deviations.

The basic simulated test scenarios consisted of five treatments and one control (Table 7). Three dose/response slope variants (steep, moderate, flat) were studied at five different growth rates (= slopes of the growth curve). The assumed factor (e.g., toxicant, light reduction) was assumed to cause an inhibition of the growth rate from 10% to 90% at steps of 20% (see Table 7). Note that this inhibition pattern was chosen for all of the scenarios, independently of slope of the growth curve, slope of the dose/response relationship, and the modelled interference factors. A basic dose/response curve was assumed for the growth rate, which followed strictly the normal-sigmoid function, so that probit analysis was the best method for determining the ECx. The selection of the "concentrations" was such that the "true" EC_{50} for the growth rate was 10.0 mg/L in all cases without interference. In the following, this EC_{50} is referred to as the "Standard value".

Table 7: Basic parameters as chosen for the simulation of algae tests; bold: average conditions as expected to be realised most frequently in testing; note that the inhibition in the growth rate was the same in all of the scenarios without additional interference factors.

	Concentration [mg/L]						**Growth rates studied [d^{-1}]**
	Control	**T1**	**T2**	**T3**	**T4**	**T5**	
%inhibition of growth rate	0	10	30	50	70	90	
Dose/Response steep	0	3.1	6.2	10.0	16.2	32.6	1.3 1.5
Moderate	**0**	**0.5**	**3.0**	**10.0**	**33.5**	**191.2**	**1.7** 1.9
Flat	0	0.2	1.8	10.0	54.2	622.6	2.1

In the algal growth model, the cell number was computed for a time period of 24 to 96 hours. The cell numbers then were evaluated by the independent PC-software, described already for the valuation of the submitted tests. For the detailed and complete series of tables and figures see Annex C (Volume III).

The following questions were examined:

- How does the experimental time, the growth rate level (=slope of the growth curve) and the slope of the dose/response curve affect the response variables and the ratio between these variables?
- Which are the consequences of shortcomings in the choice of the test concentration?
- How are the response variables affected by experimental shortcomings?
- How are the response variables affected by properties of the test substance?

5.2. The effects of experimental time, slope of the growth curve and slope of the dose/response curve on the response variables

5.2.1 The effects of experimental time on the response variable values

The first set of simulations focuses on how the experimental time, the growth rate, which is the slope of the growth curve, and the slope of the dose/response curve (steep, moderate, flat) affect the response variables and the ratio between these variables. In so doing, strictly exponential scenarios without interference factors were simulated. The values for the growth rates and concentrations were chosen according to Table 7. These settings throughout caused an exponential growth pattern as shown in Figure 41 (replace T1-T5 with the respective “real” concentrations).

Figure 60 shows the dose/response curves for growth rate[10] and the biomass integral as obtained after various experimental time periods. The moderate slope scenario was assumed for the dose/response curve. It is obvious, that in the growth rate the character of the dose/response curves and the ECx were the same, independently of experimental time (E_rC_{50} = 10 mg/L). In contrast in "Cell count" and "Biomass integral", mainly three sorts of changes were obvious during time: (1) the ECx became increasingly lower over time ($7.6 \geq E_cC_{50} \geq 0.53$; $3.2 \geq E_bC_{50} \geq 0.61$), (2) the distribution of the data points was moved to the right, towards higher values of inhibition, increasingly with time, and (3) the dose/response function was slightly different in "Cell count" and did not show the same exact fit to the normal sigmoid as was observed with "Growth rate" and "Biomass integral". The responses in the ECx of the biomass parameter, mentioned under (1), have long been known and are attributed to the exponential relationship between growth rate and cell number (Nyholm 1985). Not considered as yet, however, is the shift of the data points in the biomass parameters (more pronounced in "Cell count") and the difference in the dose/response function. Obviously, there is no unique concentration spacing, which is optimal for dose/response fitting in these response variables. The lesson to be learned here is that there is urgent need to focus on only one of the response variables, since for only one of the variables the concentration range can be optimally adjusted. Moreover, although the same "substance/factor" was constantly acting over time (e.g., a constant inhibition of photosynthesis), in the biomass parameters this factor is assessed to be more toxic after a longer experimental time.

5.2.2 The effects of slope of the dose/response curve on the response variable values

By means of Table 8 and Figures 61 and 62, the effects of the slope of the dose/response curve can be studied. Again, the growth curves were strictly exponential as shown in Figure 41, but for T1 to T5 three differently spaced concentration ranges were assumed. While no influence on the relative location of the data points on the sigmoid curve (as described above) was to be observed in the biomass parameters, the ECx markedly changed depending on the slope (Table 8). This change was more pronounced in "Cell count". At the same time the EC_{50}-ratio between the growth rate and biomass parameters increased by about one order of magnitude with decreasing slope.

Table 8: Effect of the slope of the dose/response curve on the EC_{50} of the response variables and on the EC_{50}-ratio between the growth rate and the biomass parameter.

	Slope of the dose/response relationship					
	Steep		Moderate		Flat	
	0 - 72 h	0 - 96 h	0 - 72 h	0 - 96 h	0 - 72 h	0 - 96 h
E_cC_{50}	3.7	3.1	0.8	0.5	0.3	0.2
E_bC_{50}	3.9	3.3	0.9	0.6	0.4	0.2
E_rC_{50}	10.0	10.0	10.0	10.0	10.0	10.0
Er^*C_{50}	10.0	10.0	10.0	10.0	10.0	10.0
E_rC_{50}/E_cC_{50}	2.7	3.2	12.5	18.8	34.2	60.6
ErC_{50}/EbC_{50}	2.5	3.0	10.6	16.4	27.1	50.0

The relation of the ECx-ratio for the EC_{50} and EC_{10} on slope is given in Figure 62. The slope-dependent changes in the ratio were marked also in the EC_{10}-ratio but far less pronounced than with the EC_{50}. Again, the effects were stronger in "Cell count" than in "Biomass Integral". While in the growth rate a constant ECx was obtained – according to the setting of a constant inhibition of some growth-rate proportional metabolic process -, in the bio-

[10] Due to the strictly exponential growth, the values of "Growth rate" and "Growth rate*" are identical and need not to be considered separately

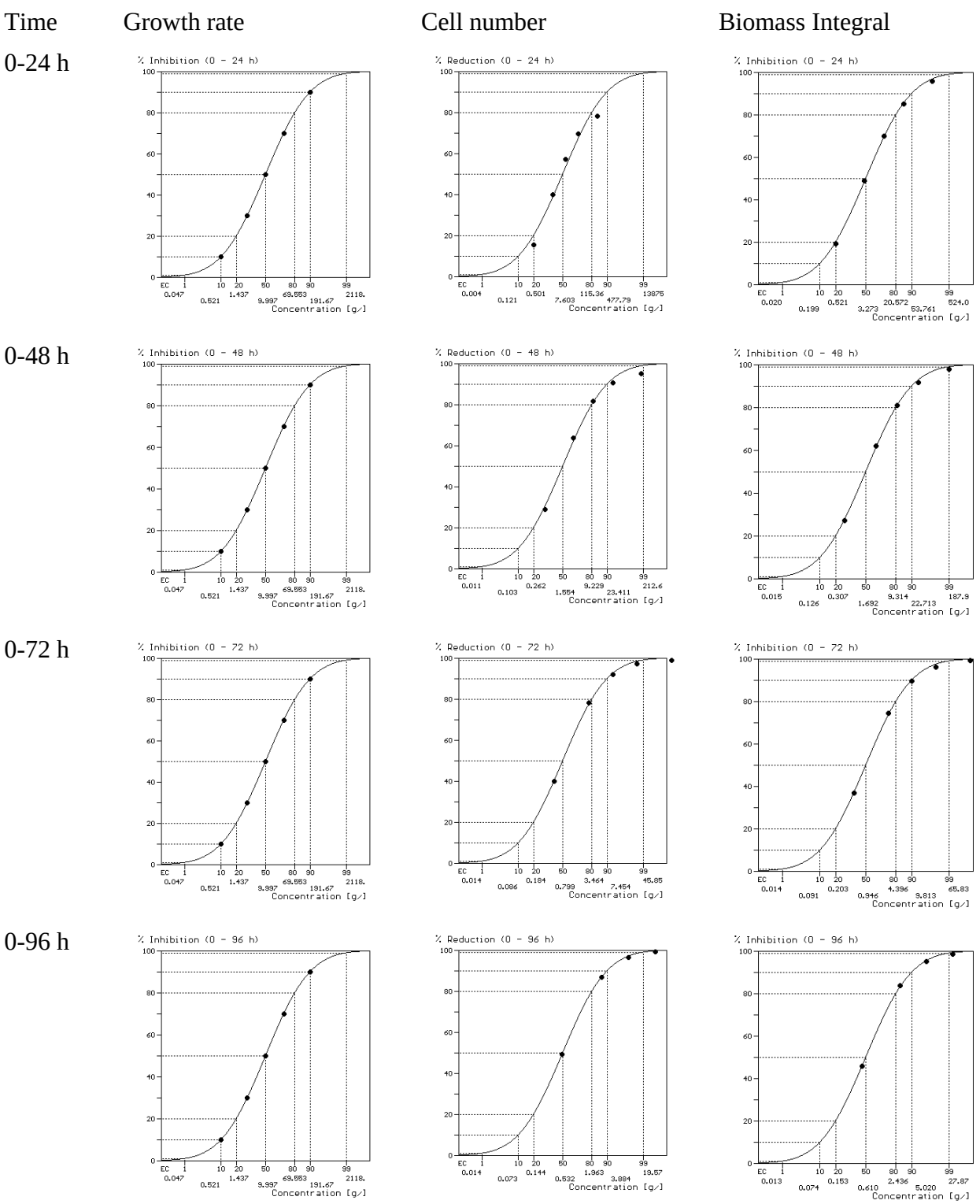


Figure 60: Moderately steep dose/response curves for average growth rate (left hand column), Cell number (central column), and biomass integral (right hand column) as obtained with strictly exponential growth ($\mu = 1.7\ d^{-1}$) after various periods of time.

mass parameters the “toxicity” is seen the less stronger the steeper the dose/response relationship is. In other words, a substance is assessed to be more toxic if its action is steadily increasing with concentration than a substance with a steeply increasing action.

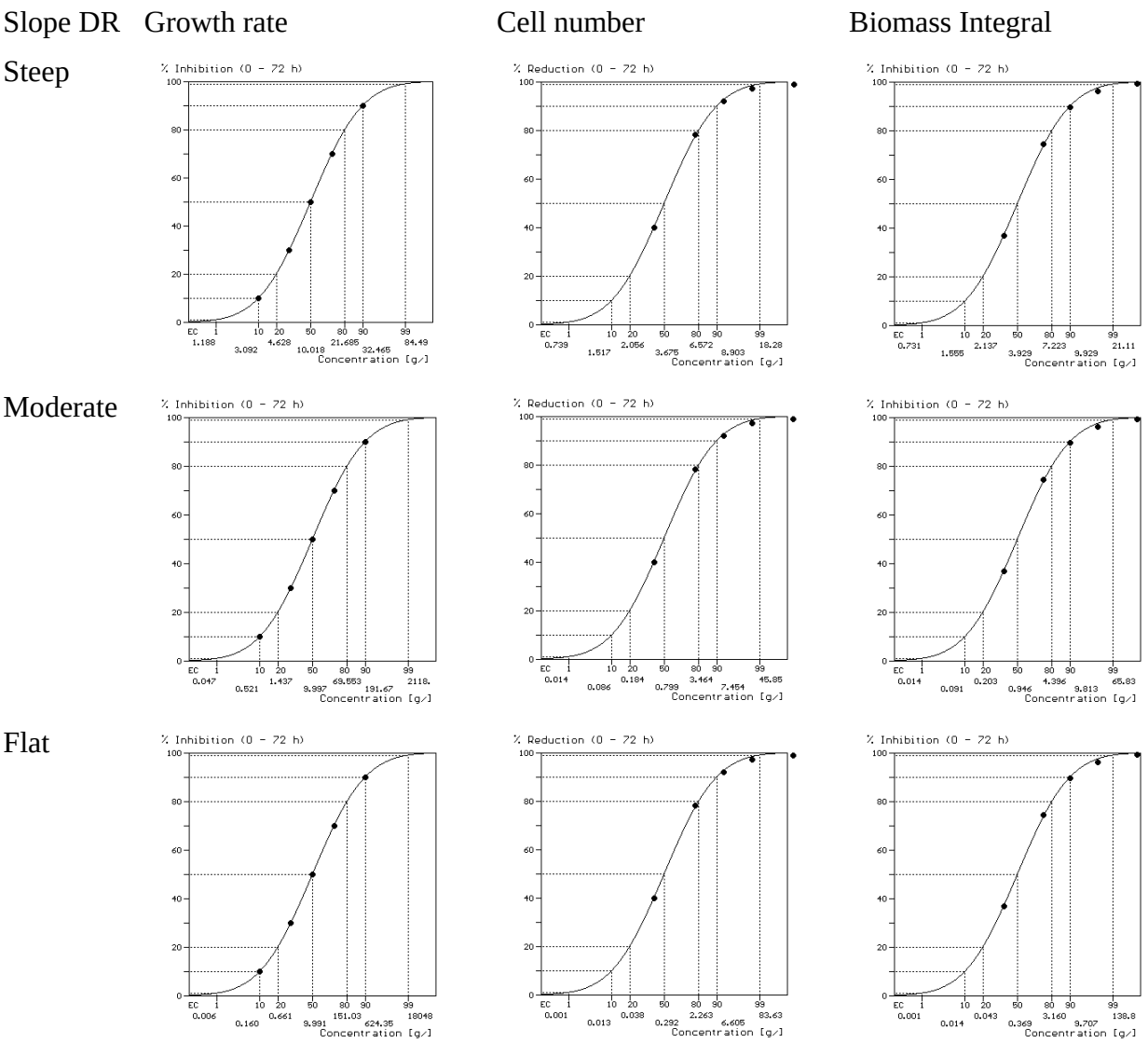


Figure 61: Dose/response curves for average growth rate (left hand column), Cell number (central column), and biomass integral (right hand column) as obtained with strictly exponential growth for 72 h (μ = 1.7 d^{-1}) for various slopes of the dose/response.

5.2.3 The effects of the slope of the growth curves on the response variable values

Additionally, the underlying growth rate was varied from 1.3 to 2.1 d^{-1}, in order to investigate how differences in growth rate, which is the slope of growth curve on logarithmic scale, are affecting the toxicity parameter ratio. Such sort of varying growth rates can occur with sub-optimal culture conditions or with the use of different algae species, exhibiting a slightly different growth rate. Again changes were observed only in the biomass parameters, while the same constant toxicity parameter values were obtained as set for the growth rate. The steeper the slope of the growth curve, the smaller was the EC_{50} in both "Cell count" and "Biomass integral" (Table 9). These effects are also reflected in the ECx-ratio (Figure 63). As with the slope of the dose/response curve, the effects were less strong in the EC_{10} and in "Biomass integral". The conclusion here is that any test condition, affecting the growth rate, influences the ECx-ratio between the toxicity parameters. Moreover, algae species with faster growth are seen as more sensitive against a substance by the biomass parameter due to their growth potential rather than due to a physiologically based higher susceptibility for the substance.

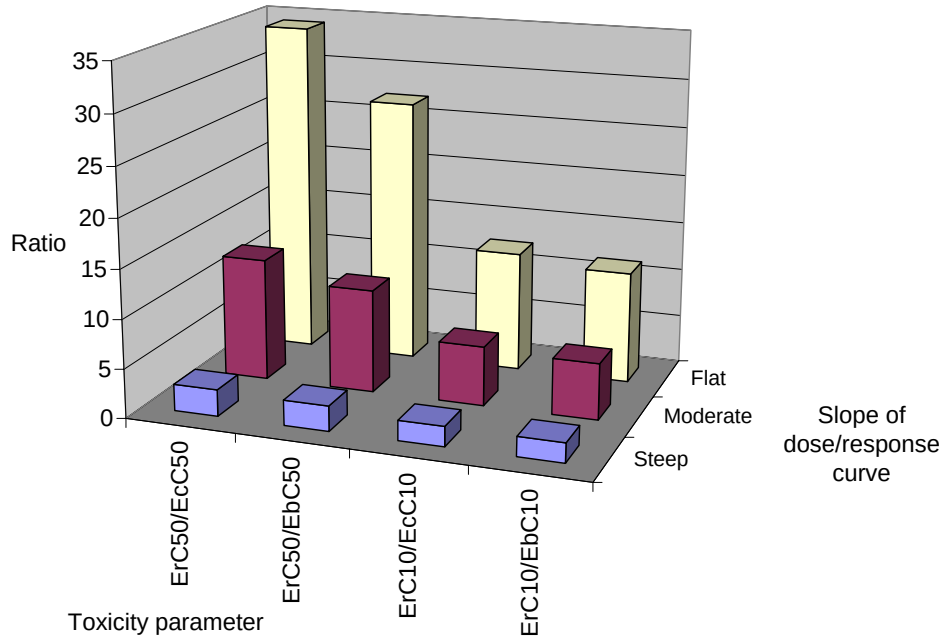


Figure 62: ECx-ratio between growth rate and the biomass parameter (cell number, biomass integral) as obtained with strictly exponential growth for 72 h (μ = 1.7 d^{-1}) and various slopes of the dose/response curve.

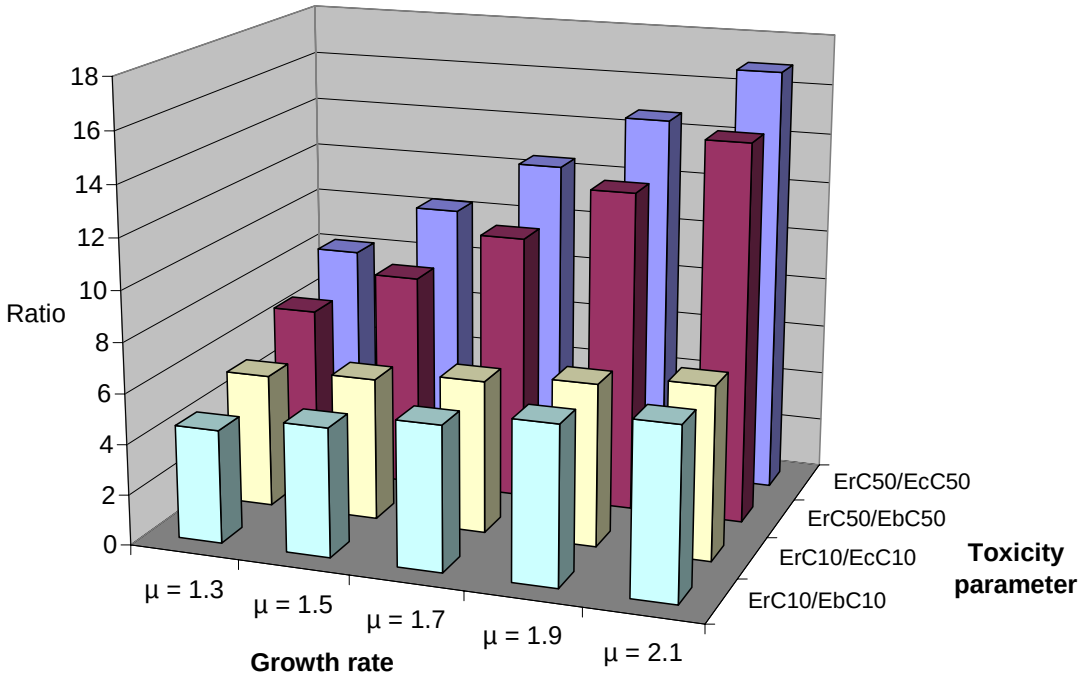


Figure 63: ECx-ratio between growth rate and the biomass parameter (cell number, biomass integral) as obtained with strictly exponential growth for 72 h at moderate slope of the dose/response curve; the growth rate varied between 1.3 and 2.1 d^{-1}.

Table 9: Effect of the slope of the growth curves on the EC_{50} of the response variables and on the EC_{50}-ratio between the growth rate and the biomass parameter. Note that the given slope μ applies for the control; in the treatments the slope was smaller due to the assumed toxic inhibition (for explanation see text)

	Slope of the growth curve (Control)				
	μ = 1.3	μ = 1.5	μ = 1.7	μ = 1.9	μ = 2.1
E_cC_{50}	1.22	0.97	0.80	0.68	0.59
E_bC_{50}	1.48	1.17	0.95	0.78	0.67
E_rC_{50}	10.00	10.00	10.00	10.00	10.00
$E_{r*}C_{50}$	10.00	10.00	10.00	10.00	10.00
E_rC_{50}/E_cC_{50}	8.17	10.32	12.51	14.72	16.94
ErC_{50}/EbC_{50}	6.75	8.55	10.57	12.75	15.03

5.3 The effects of dissimilar distribution of data points within the dose/response range on the response variables

5.3.1 Dissimilar distribution at zero variability

In Section 4.6.2, the dissimilar distribution of data points in the response range of the growth-rate and the biomass-parameter was hypothesised as a reason for extreme values in the ECx-ratio. In the previous section, a dissimilar distribution in the growth-rate and the biomass-parameter inhibition was already obvious, if the inhibition of the growth rate was ideally spaced over the whole dose/response range (e.g., see Figure 60). The dissimilarity increased with time. EC_{50}-ratios in the growth rate and biomass parameter up to 70 were already to be observed under quite ideal conditions. In the submitted tests, however, far higher ratios were found. Therefore, in the following section additional evidence will be presented on factors causing extreme EC_{50}-ratios.

The basic assumptions for the following simulations are again, that the growth was strictly exponential, but the concentration range and spacing differed from the ideal manner. In other words, the same basic growth rates and dose/response function were set as above, but it was assumed that the experimenter did choose concentrations causing either lower inhibitions than 50% or higher than 50%. These scenarios were compared with the ideal selection of concentrations causing inhibitions from 10 to 90%.

In the first of such sort of scenarios, it was assumed that the true cell numbers after 72 h were determined, i.e. no variability biased the “true” numbers. Figure 64 gives an overview over the set of scenarios tested. There are cases, in which all of the inhibitions in the growth rate were equal or below 50%, and others with all of the inhibitions greater 50%. Note, that all data points appear to be exactly modelled by the normal sigmoid response curve. As a consequence, deviations in the toxicity parameters relative to the standard run (50% inhibition is bracketed by lower and higher inhibitions) in this case can only be attributed to shortcomings in the mathematical procedures.

Figure 64 again shows that the inhibition in “Biomass Integral” was always higher than in the growth rate, i.e. the data points in the biomass were shifted to the right hand side of the dose response curve. In the considered scenarios, this leaded to extremely deviating location of the data points relative to the standard run. On the other hand, due to the absence of variability the data points follow exactly the “true” dose response curve as represented by the standard run. Will also in the extreme scenarios the “true” dose/response scenario be discovered by the mathematical procedure (i.e., probit analysis)?

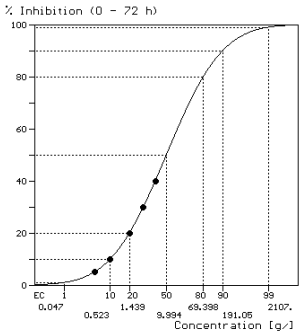
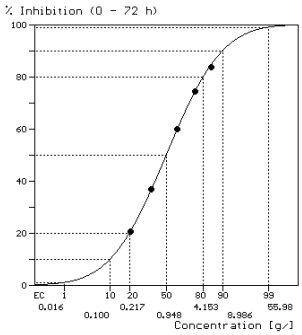
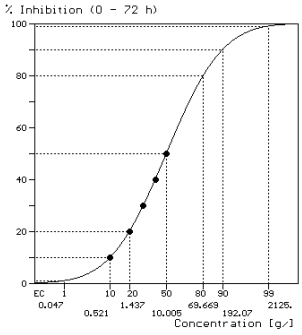
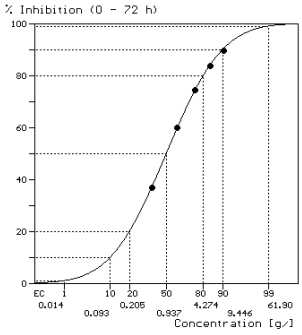
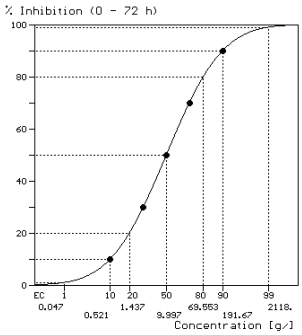
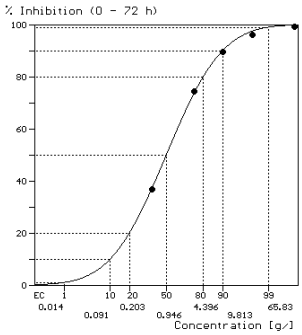
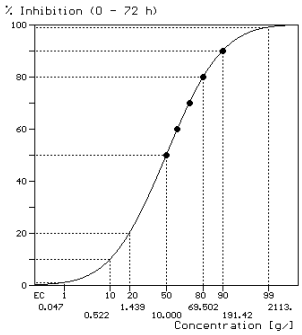
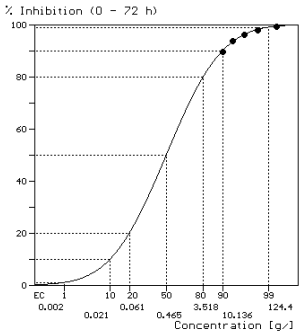
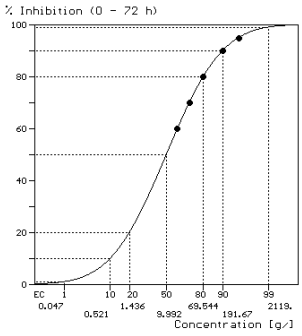
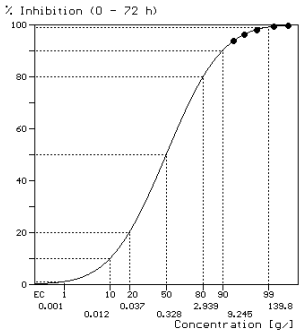
	Growth rate	Biomass Integral
Inhibition << 50%		
Inhibition ≤ EC_{50}		
Inhibition evenly spaced around 50% (standard run)		
Inhibition ≥ 50%		
Inhibition >> 50%		

Figure 65 answers these questions. Obviously, there were strong deviations in scenarios, where the growth rate inhibition was >= 50% in all data points. “Cell count” and “Biomass integral” (and in one scenario with moderate steep “Growth rate*) had markedly lower EC_{50}s than in the standard run (for the detailed results of the evaluation of these scenarios see Tables A10 to A12 (Annex A)). Only in the most extreme scenarios with moderate slope at high growth rate inhibition also the growth rate EC_{50} was lower than in the standard run (the “true” EC_{50}), but not to that extent as in the biomass parameters. In consequence, the EC_{50}-ratio tremendously increased in these cases. These effects were stronger with decreasing slope of the dose/response curve.

The lesson learned from these findings is that (1) even under ideal conditions (no variability) the mathematics is not able to find the appropriate dose/response function, if certain mistakes are made with concentration selection, and (2) that the biomass parameters are more affected in that they appear more sensitive (markedly lower EC_{50}s).

5.3.2 Dissimilar distribution and the effects of variability

From the above findings the question arises whether the variability in the cell counts, which is inevitable under real test conditions, would additionally influence the appropriate determination of the toxicity parameters and their ratios. It is quite clear that, as variability increases so will decrease the statistical precision of the computed toxicity parameter values. Whereas this general phenomenon needs not to be investigated further, the problem deserves attention whether the variability further increases the deviation of the biomass parameters, as reported above, towards higher “false” sensitivity.

In order to investigate this, scenarios were simulated, in which the coefficient of variation of the cell number varied from 5% to 20%. Figure 66 gives a sketch of the resulting variation around the dose/response curves, if in the cell counts the coefficient of variation was set to 10%. The results for all of the scenarios will be considered by means of the relative deviations from the standard run (EC_{50} ideally bracketed by the concentration/inhibition data; no variability). These relative deviations are given in Figure 67, whereas the detailed results are given in Tables A10 to A12 (Annex A). Please note, that for each scenario only one sample was drawn from the cell-number probability distribution. In other words, the deviation in the toxicity parameters and ratios are also only one possible example for such sort of deviations. In order to find the true variability-dependent deviations, per scenario one would have to be conducted between 100 and 1000 simulations, each with another random sample of cell numbers. This work had to be postponed to subsequent research. Nonetheless, the examples shown below give valuable hints on the problem of data modelling under presence of some variation.

Figure 67 shows that many of the responses in the biomass parameters became more sensitive, i.e. the EC_{50}s were lower than in the standard dose/response curve. As already demonstrated, this is already true for the scenarios, in which no variability was assumed, but the point distribution was dissimilar (Figure 65; Figure 67; S 0%, M 0%, F 0%). The deviation in

Figure 64: (Opposite page) Scenarios with dissimilar in the inhibition of growth rate and biomass integral for the moderate-slope dose/response curve; no variability was assumed; the underlying “true” dose/response curve (standard curve) is represented by the scenario in which the EC_{50} is bracketed by an equal number of concentrations/inhibitions of the growth rate; except for the standard curve it was assumed that the experimenter did not choose the appropriate concentration range and spacing; test duration was 72 h.

biomass-parameter-EC_{50} tendentiously was found to be increased with increasing slope of the dose/response curve (from S to F).

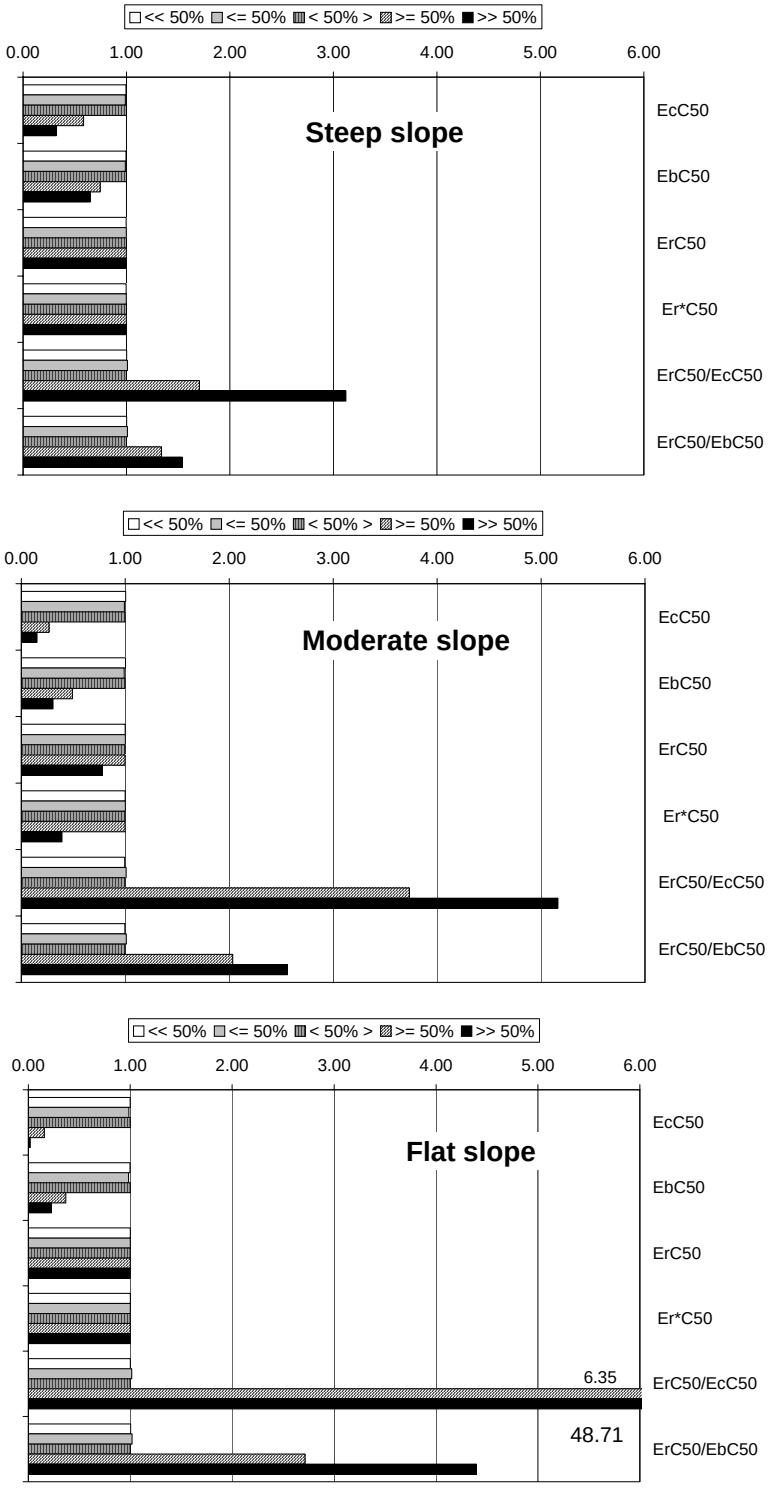


Figure 65: Factor between the scenario-EC_{50} and -EC_{50}-ratio and the standard values in various response variables as observed with scenarios dissimilar in distribution of inhibition in growth rate and biomass integral for three different slopes in the dose/response curve; no variability was assumed; the underlying "true" dose/response curve (standard curve) is represented by the scenario in which the EC_{50} is bracketed by an equal number of concentrations/inhibitions of the growth rate.

In contrast, in the growth rate the EC_{50}s varied more around the value of the standard growth curve (= the "true" EC_{50}). Nonetheless, there might on average be some tendency to higher (less sensitive) EC_{50}s, in some cases the deviations towards higher values were extreme, probably due to chance.

The resulting consequences for the ratio are given in Figure 68. Higher ratios were observed with increasing slope and increasing variability. The ratios were generally higher with the E_rC_{50}/E_cC_{50}-ratio and became extreme in the flat-dose/response curve scenarios.

Although the analysis of the variability-influence on the responses of the toxicity parameters and their ratios were based only on one single sample, each, the overall picture is that the presence of variability decreases the biomass-parameter EC_{50} more than it increases the growth rate EC_{50}. Hence the growth rate can be seen as more stable against variability than the biomass parameters. In addition to the above discovered mathematical problem in finding the true dose/response function, which cannot be influenced by the experimenter, the variability of the cell number is partly in hand of the experimenter. Thus, there should be regulations on the allowed variability of the cell number.

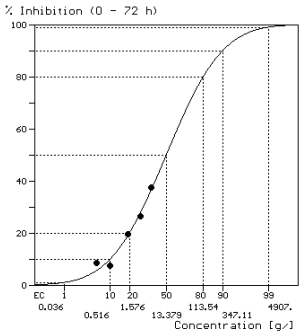
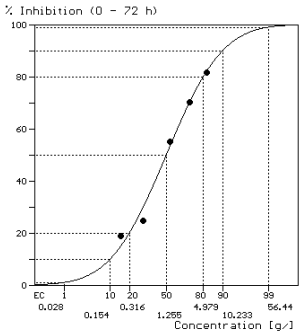
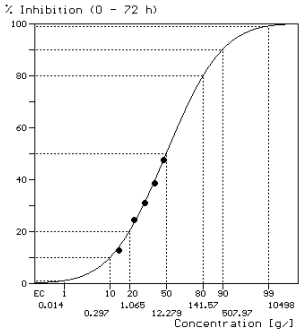
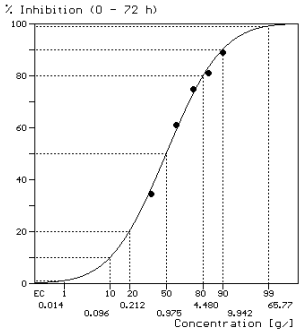
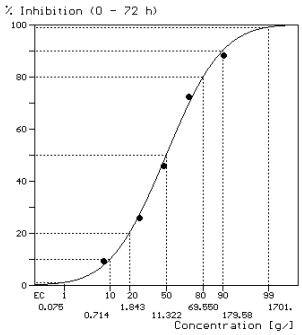
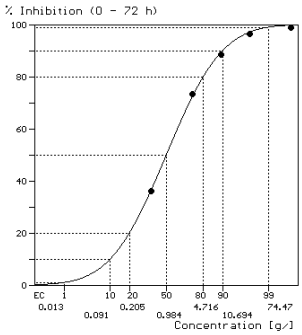
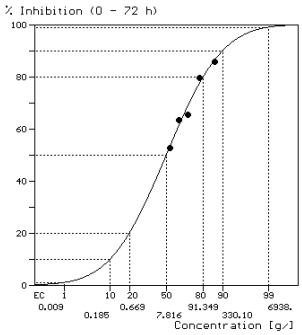
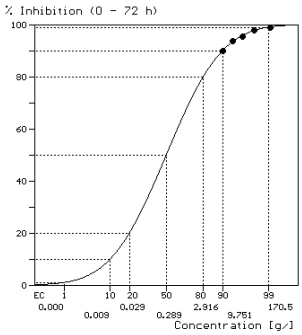
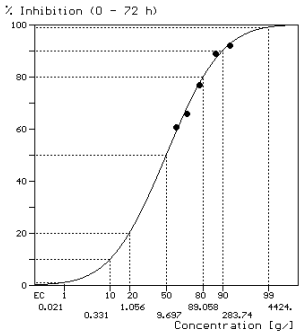
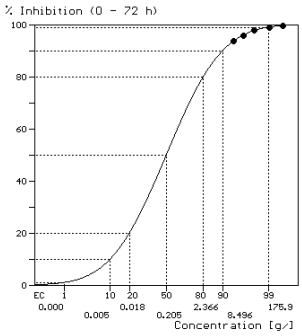
	Growth rate	Biomass Integral
Inhibition << 50%		
Inhibition ≤ 50%		
Inhibition evenly spaced around 50%		
Inhibition ≥ 50%		
Inhibition >> 50		

Figure 66: (Opposite page) Scenarios with dissimilar in the inhibition of growth rate and biomass integral for the moderate-slope dose/response curve; the variability in cell number was set to 10%; except for the scenario in which the EC_{50} is bracketed by an equal number of concentrations/ inhibitions of the growth rate; it was assumed that the experimenter did not choose the appropriate concentration range and spacing; test duration was 72 h.

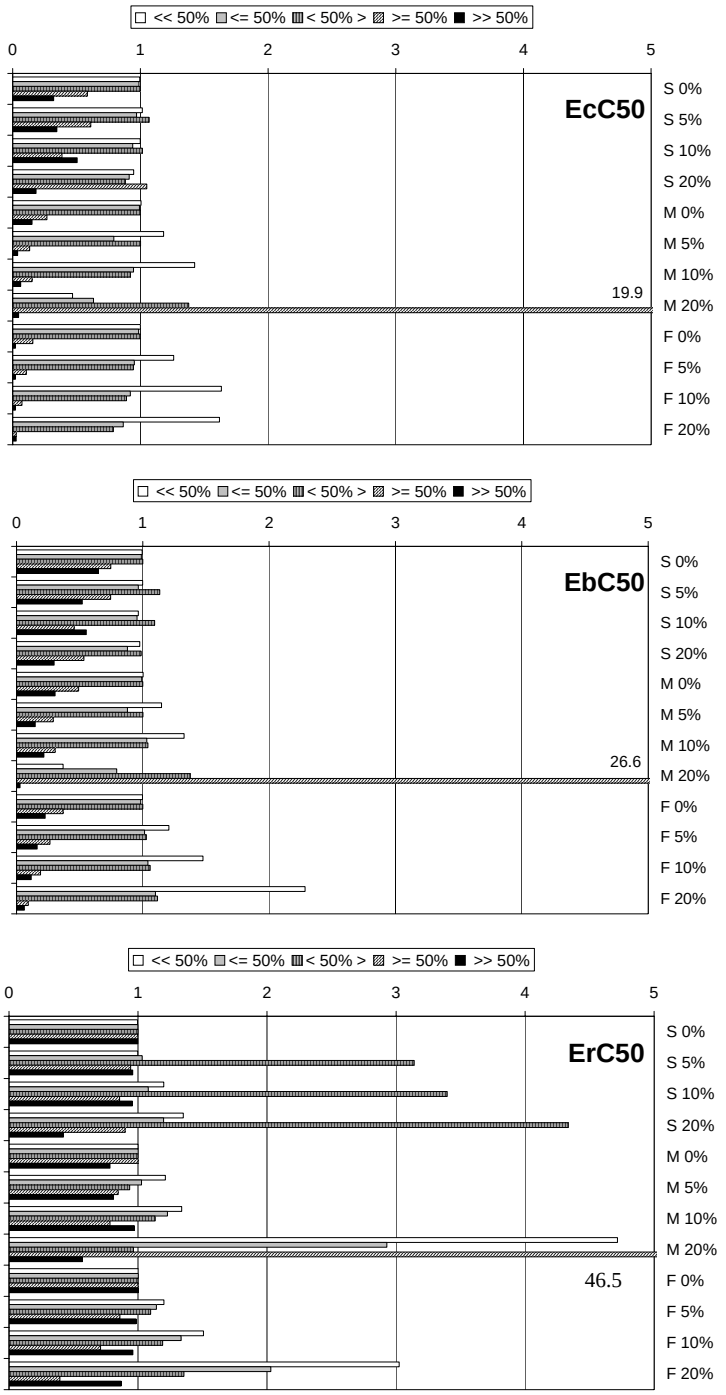


Figure 67: Factor between the scenario-EC_{50} and the standard values in various response variables as observed with scenarios dissimilar in the inhibition of growth rate and biomass integral for the moderate-slope dose/response curve; the underlying “true” dose/response curve (standard curve) is represented by the scenario in which the EC_{50} is bracketed by an equal number of concentrations/inhibitions of the growth rate with no variability; S, M, F; steep, moderate, flat slope of the dose/response curve; added percent value indicate the coefficient of variation assumed for the cell number

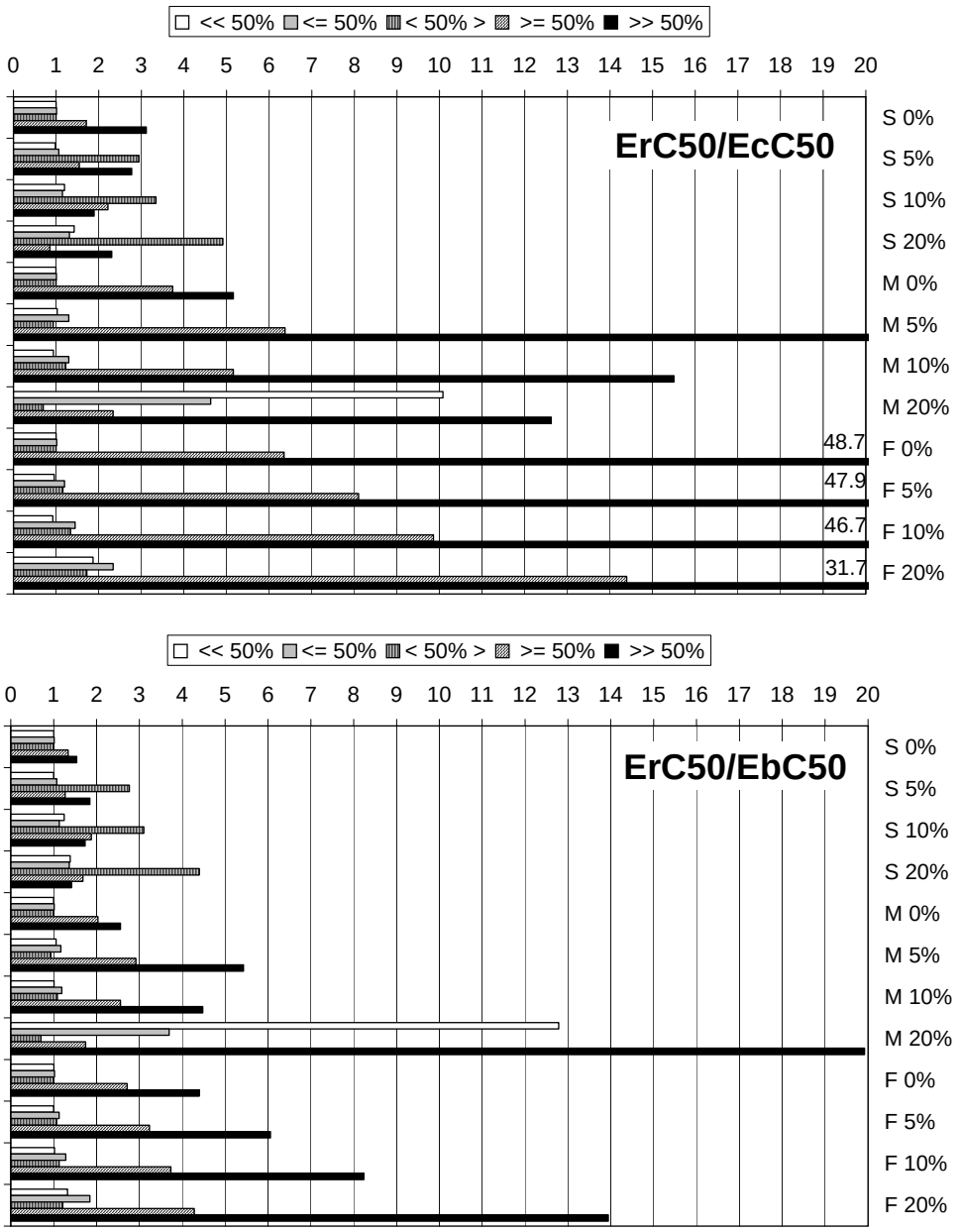


Figure 68: Factor between the scenario-EC_{50}-ratio and the standard values as observed with scenarios dissimilar in the inhibition of growth rate and biomass integral for the moderate-slope dose/response curve; no variability was assumed; the underlying "true" dose/response curve (standard curve) is represented by the scenario in which the EC_{50} is bracketed by an equal number of concentrations/inhibitions of the growth rate

5.4 Influence of the growth pattern on the toxicity parameters and their ratios

5.4.1 Introduction

While more basic relationships between influencing factors and the toxicity parameter behaviour in the various response variables were considered so far, the present section will deal with the various scenarios, identified as underlying the observed growth patterns in the submitted tests. The goal of this section is to find out which of the response variables shown a consistent and reasonable reaction on the factors acting during the test. The disturbances due to experimental shortcomings and the timely change in the toxic action are expected to be reflected in the toxicity parameter values and their relations. How to find out whether these responses are reasonable with respect to the proper toxicity assessment?

This question was answered again by simulation experiments. For all of the scenarios discussed below, the moderate-sloped dose/response curve (Table 7) with strictly exponential growth ($\mu = 1.7\ d^{-1}$) was set as the basic growth scenario. Interference factors such as nutrient limitation or increased toxic action were superimposed (added) by a positive or negative growth rate component – sometimes changing with time. To compare the resulting deviation of the toxicity parameters and their ratios, these values were compared with the basic exponential growth scenario (unlimited strictly exponential growth, Scenario 1, Figure 41; called "Standard scenario").

If a response variable could be judged as more confident or not, had to be related to the considered scenario. In other words, experimentally caused disturbances should not evoke "extra toxicity", i.e. lead to lower EC_{50}s, whereas increased toxic action should be recorded by a respective lower EC_{50}.

5.4.2 Effects of growth modification, caused by experimental shortcomings, on the toxicity parameters

A temporary disturbance by a drop in temperature or light intensity can cause a growth pattern like shown in Figure 69, which also presents the resulting EC_{50}-reactions.

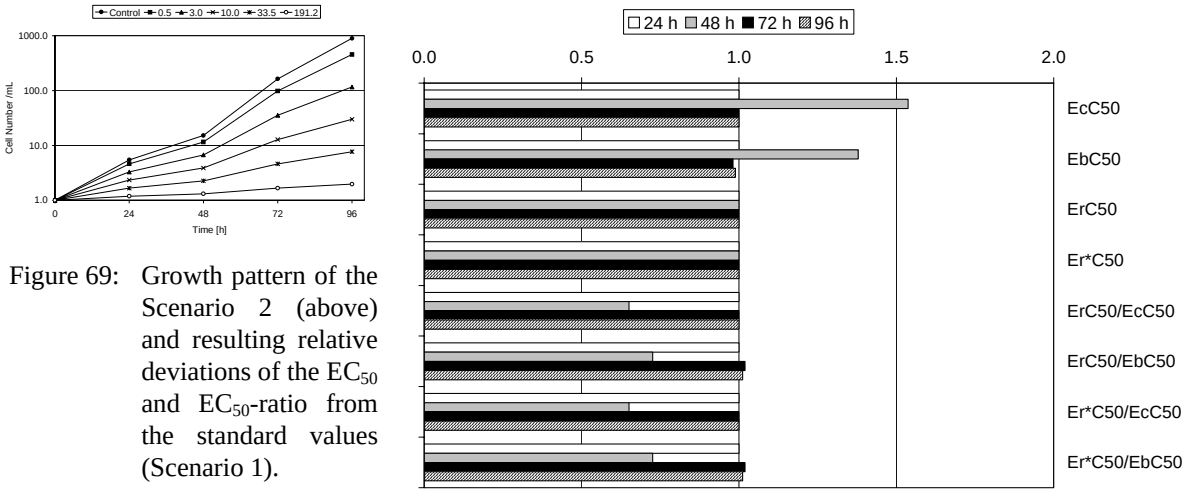


Figure 69: Growth pattern of the Scenario 2 (above) and resulting relative deviations of the EC_{50} and EC_{50}-ratio from the standard values (Scenario 1).

It is quite evident that under these conditions the biomass-EC_{50} was reacting markedly at the time when the event occurred (24-48 h). This resulted in an overestimation of the toxicity by the biomass parameters (higher E_cC_{50} and E_bC_{50}). The disturbance was "remembered" even after 72 and 96 h. In contrast, the E_rC_{50} and $E_{r*}C_{50}$ remained unaffected by the disturbance, which in this case is seen as more appropriate.

Scenario 3 (Figure 70) represents a frequently observed problem with the AGIT, the limitation of a nutrient (nutrients, carbon dioxide). This reduces the growth rate especially in the fast growing treatments and the control. Although the underlying toxicant-caused inhibition was not altered, the limitation leads to a tremendous underestimation of toxicity in "Cell count" and "Growth rate*" during the period of limitation, whereas the underestimation by "Biomass integral" and "Growth rate" was lower and in the same order of magnitude. In view of these results, the AGIT should last not longer than the nutrients can be made available. It has often been observed that limitations occurred after 72 h.

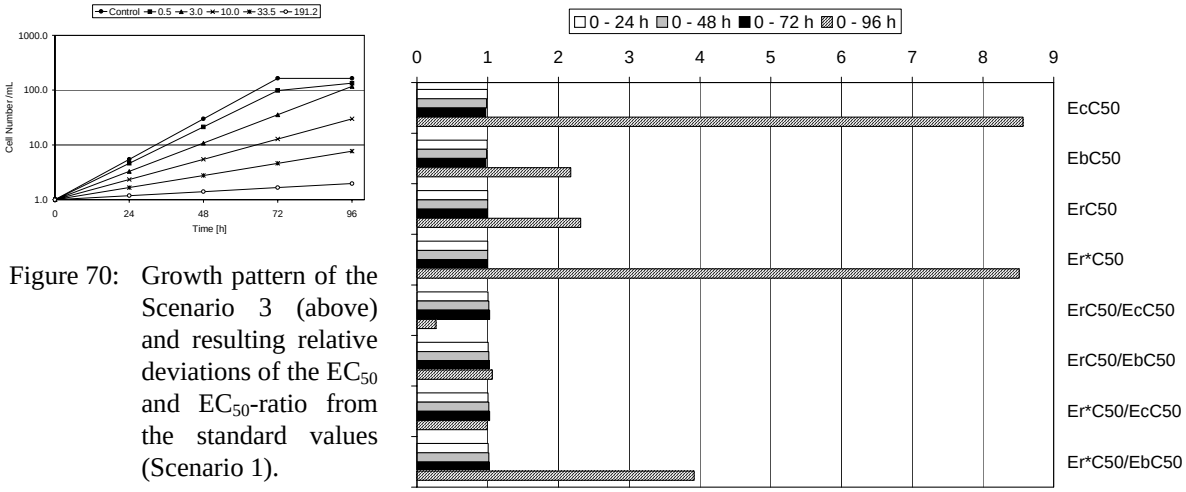


Figure 70: Growth pattern of the Scenario 3 (above) and resulting relative deviations of the EC_{50} and EC_{50}-ratio from the standard values (Scenario 1).

Figure 71 exemplifies an initial lag-phase in algal growth. Although the conditions would allow immediate exponential growth, this is not possible due to physiological reactions of the algae. The effect on the EC_{50} is marked in all response variables, results in lower EC_{50}s, decreases with time and decreases more in the growth rates. This lead to an marked increase in the EC_{50}-ratios – especially if the section by section growth rate is involved. Under appropriate pre-culture conditions such sort of effects can easily be prevented. In view of the marked overestimation in toxicity at a moderate expression of the lag-phase, tests like these should be rejected.

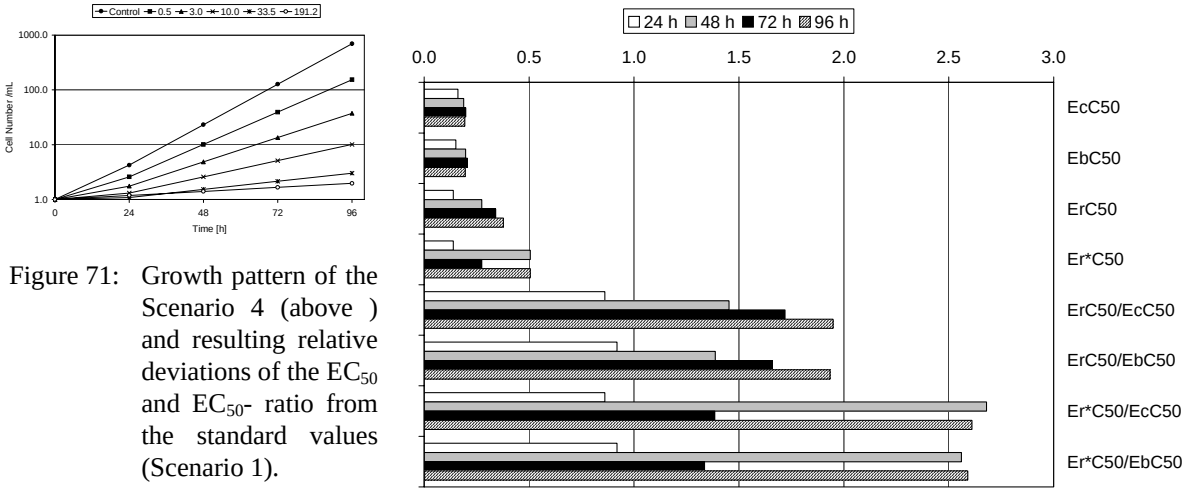


Figure 71: Growth pattern of the Scenario 4 (above) and resulting relative deviations of the EC_{50} and EC_{50}- ratio from the standard values (Scenario 1).

In the following scenario, the lag-phase is more pronounced and at higher concentrations negative growth occurred in the initial phase (Figure 72). The reaction of the response variables differed. “Cell count” proved to underestimate toxicity (higher EC_{50}), while this was more pronounced at time of the lag-phase. Except for the first time period this also applies for “Biomass integral”. Both types of growth rate on average markedly overestimated toxicity. The resulting EC_{50}-ratios were smaller than in the Standard Scenario.

In view of the same overall wrong estimation of toxicity as reported for the previous scenario, the same conclusion applies here: Tests with pronounced lag-phases lead to false estimations of toxicity.

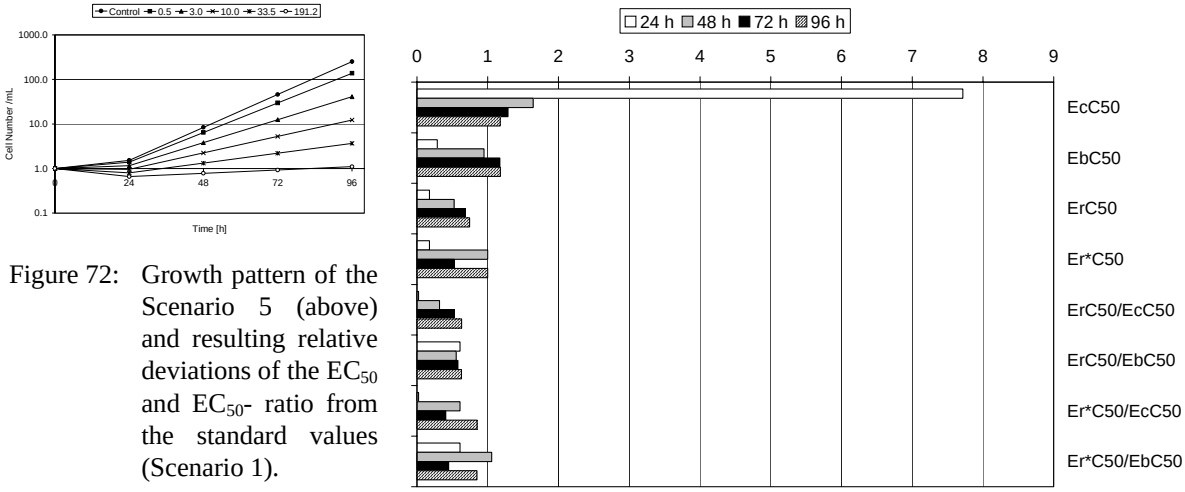


Figure 72: Growth pattern of the Scenario 5 (above) and resulting relative deviations of the EC_{50} and EC_{50}- ratio from the standard values (Scenario 1).

Scenario 6 describes an experimental error probably occurring rather rarely, although for this error strong evidence was presented above: The assumption of a false initial cell density. Except for “Cell count”, this leads to an underestimation of toxicity, which is most pronounced for the first time period. Because this growth pattern is very characteristic and thus can easily be recognised, those tests should be rejected.

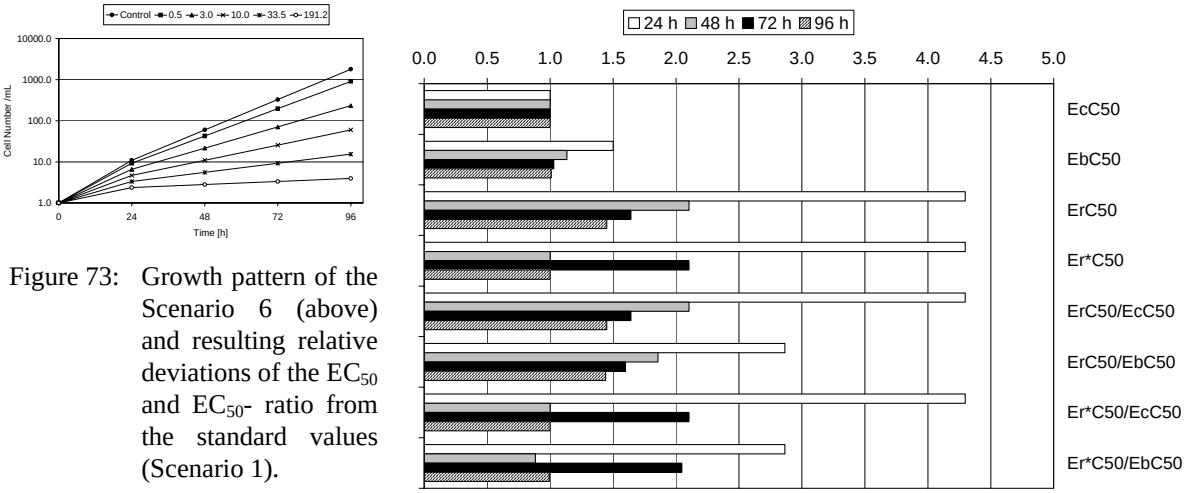


Figure 73: Growth pattern of the Scenario 6 (above) and resulting relative deviations of the EC_{50} and EC_{50}- ratio from the standard values (Scenario 1).

In Scenario 7 (Figure 74), it was assumed that the initial cell number was lower than suggested, which causes a lag-phase like growth pattern and a principally similar response as in

the lag-phase scenario 5 (Figure 72). Indeed there are no means to distinguish between these two scenarios, but both are due to experimental errors and, since the effects on "toxicity" are marked, they should be not considered for a reasonable toxicity estimation.

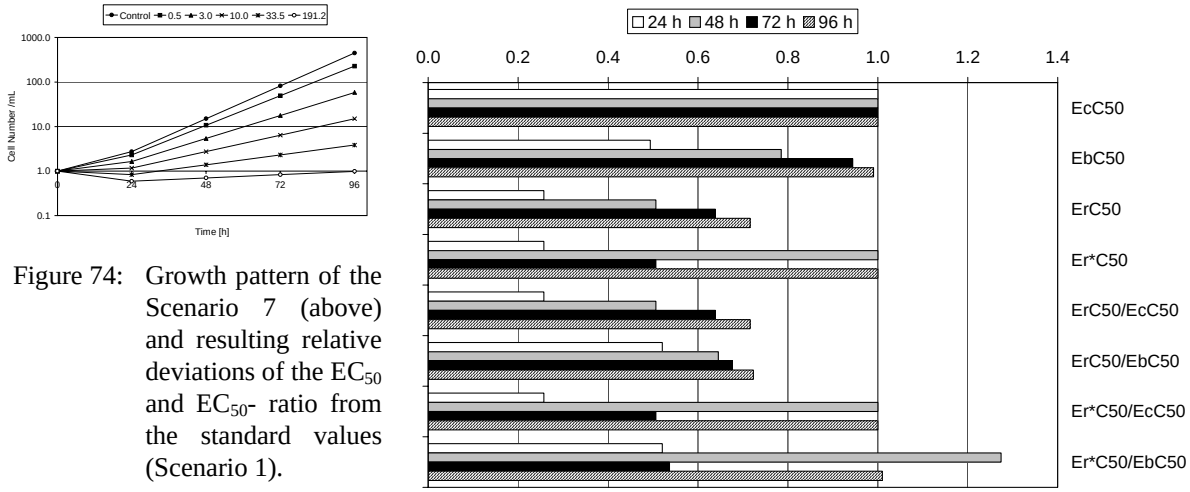


Figure 74: Growth pattern of the Scenario 7 (above) and resulting relative deviations of the EC_{50} and EC_{50}- ratio from the standard values (Scenario 1).

Summarising, the results with scenarios, simulating experimental shortcomings and errors, in most cases strongly affect the correct estimation of toxicity. Submitted tests should be watched for those types of errors and be rejected if necessary.

5.4.3 Effects of growth modification due to test-substance effects

Scenario 8 represents an initial lag-phase due to effects of the test substance rather than due to the pre-culture conditions (Figure 75). Possible modes of toxic action are mentioned in Section 4.3.4.1. The biomass parameters reacted more sensitively than the growth rates, which resulted in an increase in the EC_{50}-ratio up to values above 100.

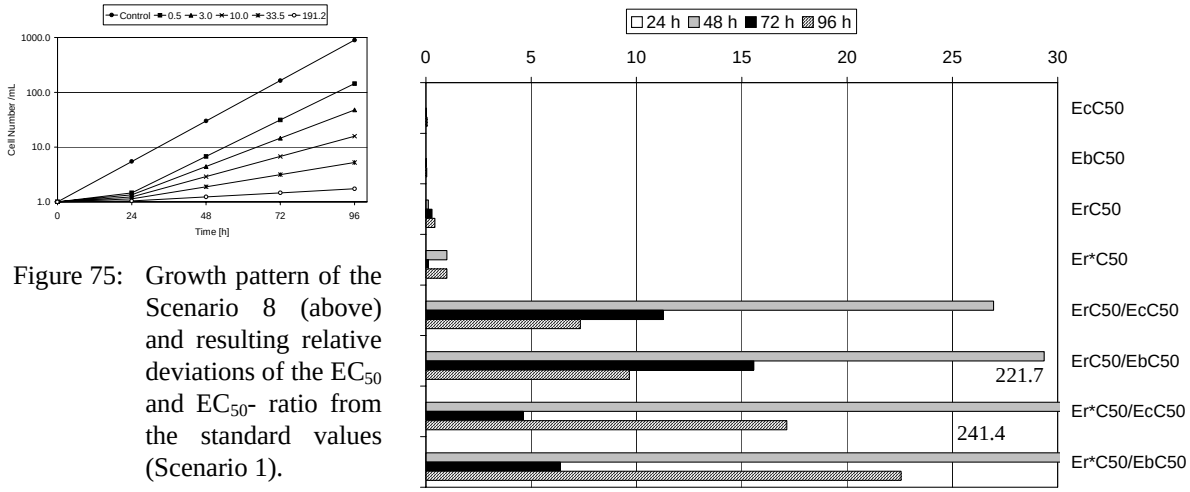


Figure 75: Growth pattern of the Scenario 8 (above) and resulting relative deviations of the EC_{50} and EC_{50}- ratio from the standard values (Scenario 1).

In Scenario 9 (Figure 76), a temporary toxic action was assumed, lasting until 48 h; thereafter the growth proceeded at the normal uninhibited rate. The temporary toxic action is generally assessed to be lower than in the Standard Scenario with continuous toxic action. However, the differences in the biomass and growth rate parameters are tremendous. Whereas all parameters correctly reflect the same toxicity as in the Standard scenario until 48 h, after 72 and 96 h the biomass parameters indicate a much higher underlying toxicity than the growth rates,

which assess more the recovery event. The resulting EC_{50}-ratio was markedly higher than in the standard scenario.

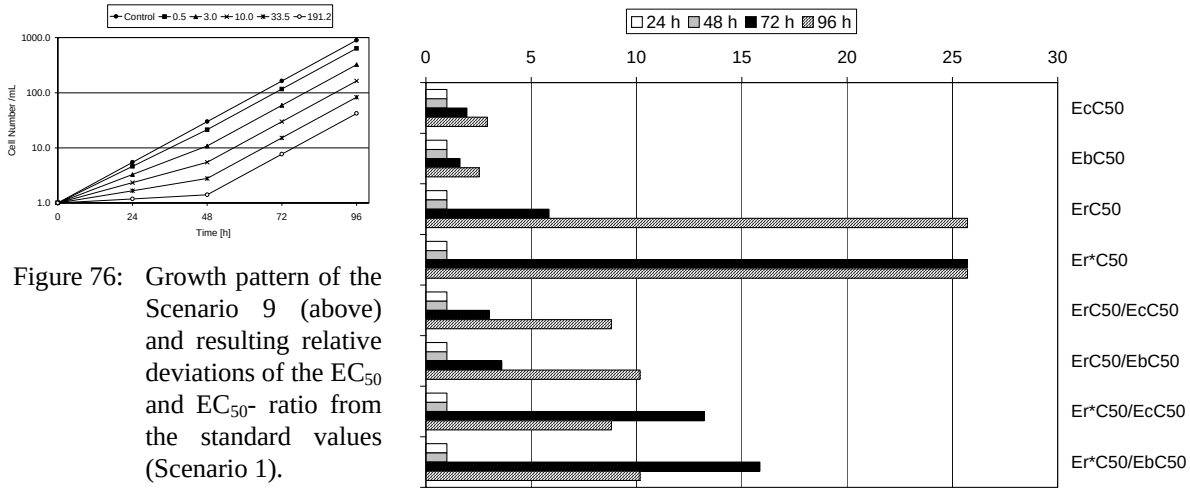


Figure 76: Growth pattern of the Scenario 9 (above) and resulting relative deviations of the EC_{50} and EC_{50}- ratio from the standard values (Scenario 1).

In Scenario 10 (Figure 77), the toxicity increased during the test duration. This is detected by all of the response variables, less by the biomass parameters, more by the growth rates. The resulting EC_{50}-ratios were lower than in the Standard scenario. It has to be discussed which of the response variables assesses the emerging toxicity more reasonable.

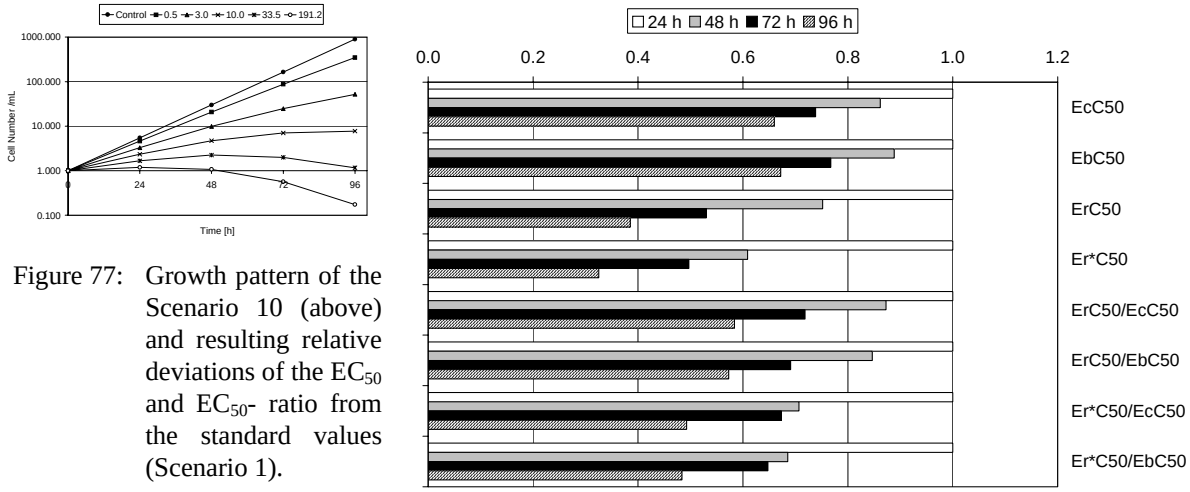


Figure 77: Growth pattern of the Scenario 10 (above) and resulting relative deviations of the EC_{50} and EC_{50}- ratio from the standard values (Scenario 1).

The algae growth can be promoted sometimes. Scenario 11 (Figure 78) represents a permanent slight promotion of the growth throughout the experimental time. This leads to higher EC_{50}s - especially in the biomass parameters, which assess the underlying toxicity in the inhibiting concentrations less then the growth rates. Again, the growth-rate response is closer to the response in the Standard scenario than the biomass parameters.

In the last scenario studied, the promotion was to be assumed to be temporary during the first experimental phase (Figure 79). As above the biomass parameters assessed this effect more positively than the growth rates, which showed a higher sensitivity than in the Standard scenario, but basically corresponded more with the Standard scenario.

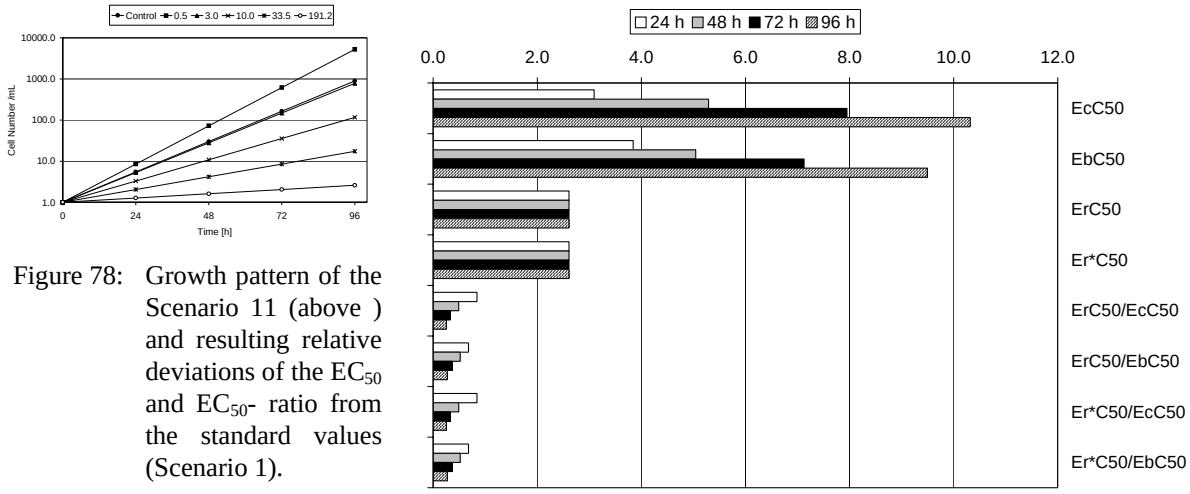


Figure 78: Growth pattern of the Scenario 11 (above) and resulting relative deviations of the EC_{50} and EC_{50}- ratio from the standard values (Scenario 1).

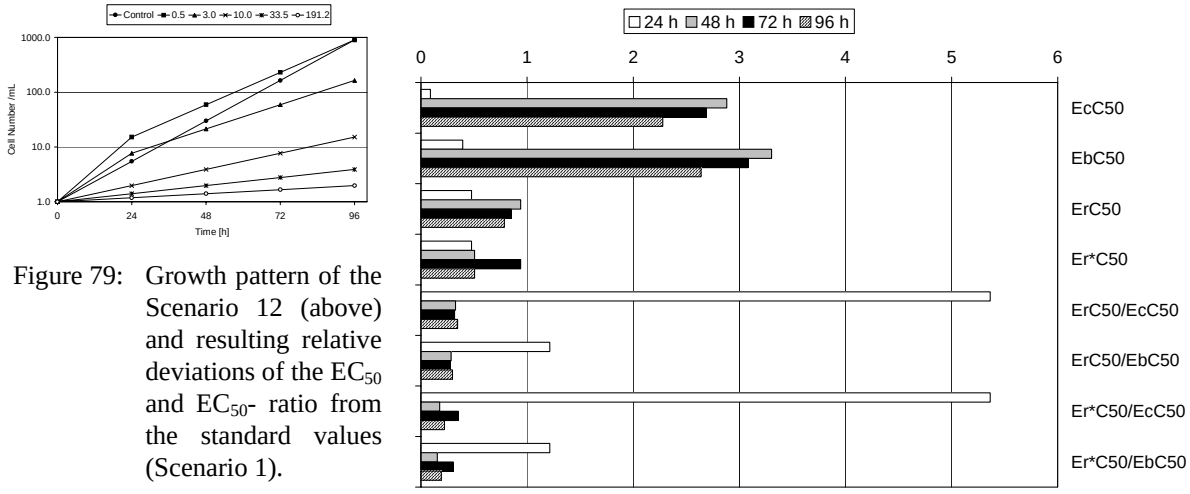


Figure 79: Growth pattern of the Scenario 12 (above) and resulting relative deviations of the EC_{50} and EC_{50}- ratio from the standard values (Scenario 1).

5.4.4 Conclusions from the simulations

The simulations were done on the basis of the growth rate and an exponential growth pattern, which is the easiest approach to simulate population growth. The biomass (cell number) observed over time is the result of a starting density and a certain growth rate during that time. The prime benefit of simulation experiments like conducted by the present study is that the basic toxicity was known and was equal in all scenarios – except for those in which the toxicity itself was changed (Section 5.4.3). Disturbances or changes in toxicity were added as a loss rate or an additional rate changing the underlying basic growth rate. The basis toxicant-induced response of the growth rate was modelled to follow exactly the normal sigmoid function, thus could be appropriately evaluated by the probit analysis. Also this basic response was kept equal in all of the scenarios. One might argue that this way of simulations is growth-rate leaded and probably would automatically lead to "better" results for the growth rate. This argument, however, does not hold, since under the premise of exponential growth the relation between the growth rate, cell number and time is fixed and thus the same relationships would be observed also with different model approaches, which, however, for such sort of growth simulation do not exists, since these are not reasonable.

The simulated cell numbers than were evaluated as if they had emerged from a real test. The EC_{50} of the average growth rate was set to 10 mg/L independent of the slope of the dose re-

sponse curve. In almost all of the scenarios the correct EC_{50} was indeed monitored by the evaluation software, which did not "know" the settings. So it was possible, to compare the EC_{50} obtained with various scenarios with this standard-EC_{50} of 10 mg/L. The similar is true also for the biomass parameter EC_{50}s, which were compared with their standard-values (see Scenario 1, Table 8). This approach allowed showing, which of the response variables and which of their ratios reacted more strongly under the considered scenario than others, which is demonstrated by a higher percent deviation from the standard than in other variables.

The simulations confirmed the hypothesis that it is impossible to design a test such that for all of the response variables the concentration range is optimal for the dose/response model. Hence it appears reasonable to focus on one of the response variable and find the optimal design for this variable.

The conclusions from the studied deviations from the standard scenario is, that as a rule the EC_{50} of "Growth rate", the average growth rate, did show less deviation from the standard than any other variable, thus reacted as the most confident estimate of the "true" toxicity. "Cell count" and thereafter "Biomass integral" and "Growth rate*", the section by section growth rate, reacted more strongly ("excitedly"). The biomass parameters indicated even a substantial toxicity under the non-toxic disturbances. In scenarios, in which the toxicity itself was changed, "Growth rate" appeared to react more reasonable. One of the most striking results was that, although the same basic toxicity action was assumed, in the biomass parameters the toxicity was seen stronger if the slope of the dose/response curve decreased and the growth rate level increase. This in principle also is predicted by Eq. 5 (Nyholm 1985). It has to be questioned whether this is appropriate and is against a reasonable perception.

6. Final conclusions

6.1 Test design and experimental conditions

In a substantial number of tests, there was strong evidence for shortcomings in the preparation and conduct of the AGIT, ranging from inappropriate pre-culture of algae, leading to lag-phases, to bad selection of concentration range and spacing. Except for a temporary inconstancy, e.g. in temperature or light conditions, all of the discussed resulting disturbances of growth impeded the correct assessment of the toxicity, since this was under- or overestimated. In cases with dissimilar distribution of concentration/inhibition data points on the dose/response curve, mathematics was not able to find the appropriate dose/response function thus leading to false estimates of toxicity. Although this has been exemplified only by the probit method, it has to be expected that this will be a general problem also with other statistical functions, since the basic relationship between biomass and growth and the test design are probably the cause. Nonetheless, it should be investigated whether statistical methods other than probit might be less susceptible under these conditions.

However, because the mentioned shortcomings to a great deal could be prevented by the experimenter, tests with such sort of design and disturbances should not be accepted.

6.2 Growth pattern

The basic growth pattern was exponential in many cases – at least for some period of time. It is inevitable that toxicants, exhibiting a timely changing toxicity, will also change the strict exponential pattern. Other deviations were caused by experimental shortcomings. But, there are no reasons to move to a growth model for algae other than the exponential one.

6.3 Statistics

For use with parametric statistical testing, both growth rate variants and "Log(Cell count)" proved to be appropriate rather than "Biomass Integral". With statistical testing of "Cell count", the log-transformation together with a parametric test should be performed rather than a non-parametric test. For the determination of a NOEC, Williams test procedure appeared as more appropriate than Dunnett's test or the non-parametric Bonferroni-U test.

With respect to modelling the dose/response function, the weighted maximum-likelihood regression analysis with probits, logits or the Weibull-transforms should be performed or a non-linear regression procedure. Also with dose/response modelling, a low variance as observed with "Growth rate" is beneficial in finding the appropriate function and in computation of confidence limits. For practical reasons, it should be avoided to confront practitioners with a couple of statistical approaches from which one has to be selected by him. However, it should be further studied whether approaches other than the maximum-likelihood probit analysis are on average more reasonable in practice, so that one of these should be prescribed for the experimenter.

From many of the results, differing in the submitted reports and the present study, there is clear evidence that the statistical methods for determination of the toxicity parameters should be standardised and prescribed.

6.4 Toxicity parameters

If the NOEC shall be replaced by some ECx, the EC_{10} appears more appropriate than another ECx, where x > 10. Extreme EC_{50}-ratios between the growth rate and the biomass parameter are clearly due to an inappropriate choice of the concentration range. This results in an inappropriate extrapolation of the EC_{50} and strongly biased estimations of its value.

The ECx-ratio between the growth rate and the biomass parameter is lower, if the most sensitive ECx of the growth rate from the investigated time periods is used and/or if the section by section growth rate is inserted. The EC_{10}-ratios are lower than the EC_{50}-ratios.

6.4 Response variables

The use of different response variables for one endpoint, the algae growth, in real and simulated tests resulted in different toxicity parameter values, which is problematic for a proper assessment of the toxicity of a test substance. It has been shown by the present study that the same concentration range is not optimal in all of the response variables. Therefore, it is desirable to select only one of the variables and optimise the concentration range for this variable.

The criterions for selection the appropriate response variable can be different: relevance for population dynamics, sensitivity, statistical confidence. In the scientific literature, the growth rate is favoured as a population parameter of prime importance and this is seen as a reason to select the growth rate as the alone response variable in a test. The cell number, generated during a certain period of time, is a biological variable, which at first sight can easily be interpreted. However, at given cell density the physiological state can be different: active or inactive. Observations in the test can hardly be transferred to the field, where the standing crop biomass is the result of a steady state of algal growth and losses due grazing.

The biomass integral is a mathematically built variable for which direct biological meaning cannot be given. Of course, it is proportional to the cell number.

Material and energy flow in a pelagic community is characterised by gain and loss rates. The growth rate appears to be the most appropriate variable to estimate gain rates, and thus is required for many mathematical population or ecosystem models. Although growth rates, as obtained in the laboratory, cannot be directly extrapolated to the field with respect to their magnitude, the relative inhibition of the growth rate seems to be transferable to field conditions, provided the toxicant is present at the same concentration.

The conclusion from this is that for a use of test results in ecological assessments (sensu strictu) it is inevitable to determine the toxicity dependence at least of the growth rate.

It was quite evident that the biomass variables react more sensitive, i.e. show lower values of the ECx. This might be seen as an advantage for their use with regulations. However, clear evidence was presented that the toxicity monitored by these variables was strongly affected by experimental conditions and test design. Even under ideal exponential growth and ideal range and spacing of concentrations, toxicity was strongly influenced by the slope of the dose/response curve and the level of the basic growth rate. Whereas in the first case one could argue that the slope of the dose/response curve is an immanent property of the test substance, the direction of the toxicity increase, suggested by the biomass variables, appears to be questioned. In the second case a physiologically based property of the algae as is monitored together with the substance toxicity, either increasing or decreasing it. Summarising, the biomass parameters appear to monitor a substantial amount of “pseudo”toxicity. In contrast, at least the average growth rate was not susceptible for these phenomena.

One of the problems, put forward with the average growth rate, is that it is determined by two points, from which one, the initial cell number, cannot be measured accurately. It has to be recommended that the computed number from the dilution of the stock culture should be used, which appears more accurate as counting the number (see also Nyholm 1985). With respect to the statistical performance, the average growth rate, as obtained from the submitted tests, proved to be superior to all of the remaining variables due to its high degree of normal distribution, variance homogeneity, and lower coefficients of variation and thus will cause fewer problems with statistically testing and data modelling.

Overall, there are sound reasons ranging from the ecological relevance, correct toxicity assessment to statistical power for the preference of the average growth rate as the basic response variable for algal growth and for the consistent assessment of toxicity.

7. References

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ANNEX A

A1 Results on basic test parameters

A1.1 Statistical distribution and variance homogeneity of response variables - prerequisites for parametric test methods

The statistical properties of response variables were investigated for the treatment and control replicates at all measurement intervals performed in a test (Figure A1). The normal distribution and variance homogeneity are prerequisites for the powerful parametric tests such as the Dunnett test and Williams test. The Dunnett test apparently is the most frequently used test for the determination of a NOEC. All treatment and control replicates were examined for each measurement interval. Generally, the proportion of normally distributed treatments and controls was higher than 80%, except for those of the first measuring interval, showing about 70% normal distribution. The degree of normal distribution showed an increasing tendency over time. Among the response variables the order in degree of normal distribution in controls was: Log (biomass integral) > Log (Cell count) > Biomass Integral, Growth rate > Cell count and Growth rate* (Figure A1 g). The proportion of normally distributed data sets was about 5% lower, in case all treatments and controls were considered (Figure A1 h), indicating that for the tests under consideration marked effects of the test substances on the statistical distribution did not occur. The prerequisite “normal distribution” is fulfilled in about 80% of cases.

In contrast to the normal distribution, which can be separately tested for each control and treatment data set, the homogeneity of variance is examined using all of the control and treatment data sets in one test (Figure A2). So the number of data sets tested is lower than with the normal distribution. “Cell count” and “Biomass integral” showed the lowest homogeneity, decreasing over time, whereas in “Growth rate” and “Growth rate*” the homogeneity was highest without a clear tendency over time. The log-transformation increased the homogeneity of data sets only in “Cell count” (by about 20%), whereas a marked decrease in homogeneity was found in “log (Biomass integral)” (Figure A2 d, g). Overall, in “log (Cell count)” and “Growth rate” the highest homogeneity was to be observed (Figure A2 g), followed by “log (Cell count)”, and “Growth rate”, all ranging between 70 and 80% homogeneity. In contrast, in “Cell count” and “Biomass integral”, homogeneity was about 50 to 74% and thus markedly lower.

Summarising, the prerequisites “normal distribution” and “variance homogeneity”, as demanded by parametric statistical methods, can be seen as fulfilled to a great extent by both growth rates and “log (Cell count)”. Here the Dunnett’s and Williams tests will lead to an appropriate determination of a NOEC. In the remaining test data sets, non-parametric test methods would be appropriate, which however are less powerful, reject the Null-Hypothesis at greater differences, and thus can lead to higher values of the NOEC than with parametric test methods in some cases. However, it should be considered that the number of replicates, currently investigated in common AGITs, is relatively low and despite of the fulfilment of the prerequisites does often not allow conducting powerful statistical testing.

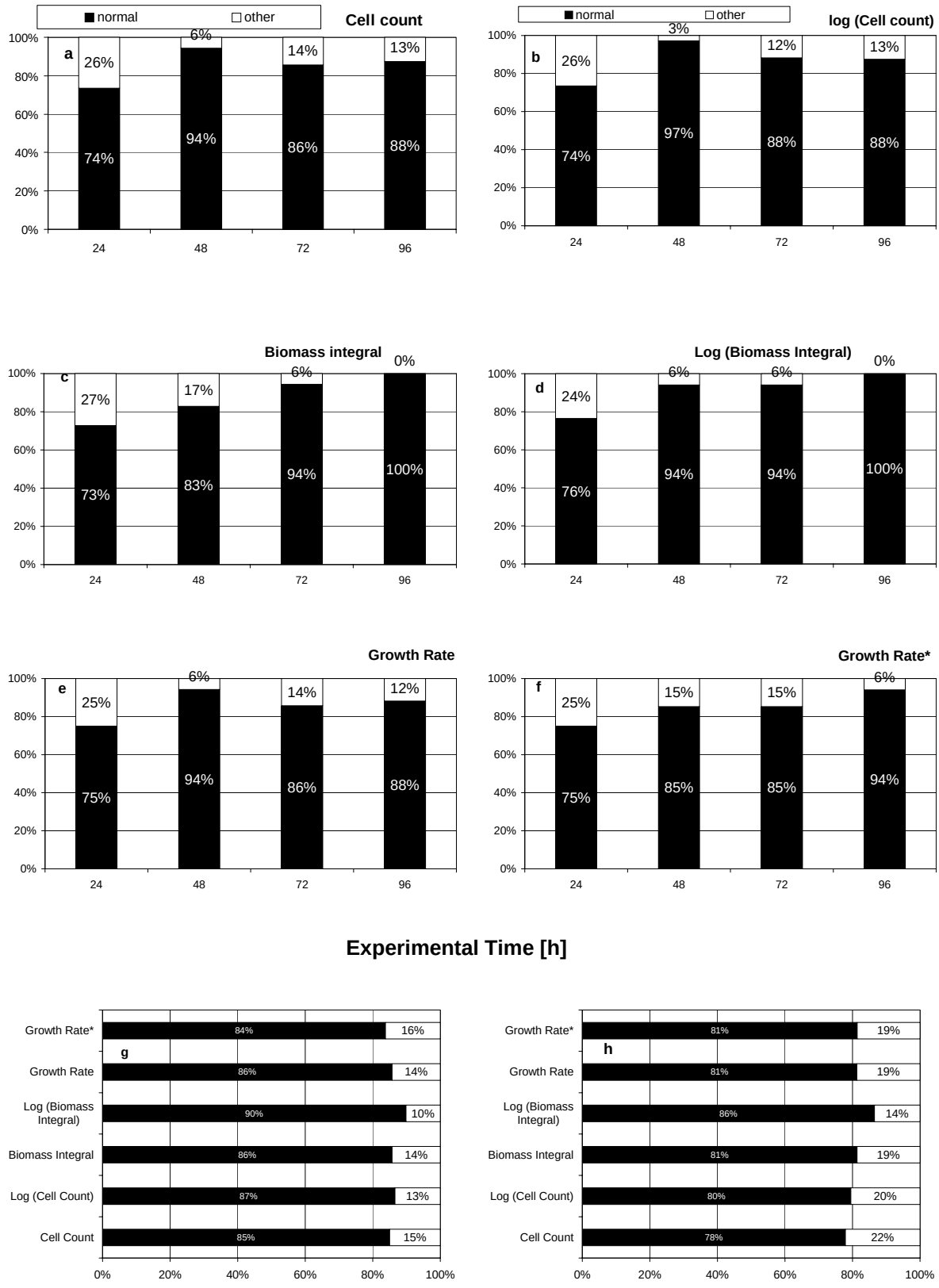


Figure A1: Results of the R/s-test procedure, examining the normal distribution of replicate values of the response variables “cell count” (a), “log (cell count)” (b), “biomass integral” (c), “log (biomass integral)” (d), “average growth rate” (e) and section by section growth rate (“Growth rate*”) (f) in controls (α= 0.05; on average n = 35 per response variable);g: all controls lumped over time (on average n = 109 per response variable); h: all controls and treatments lumped over time (on average n = 653 per response variable)

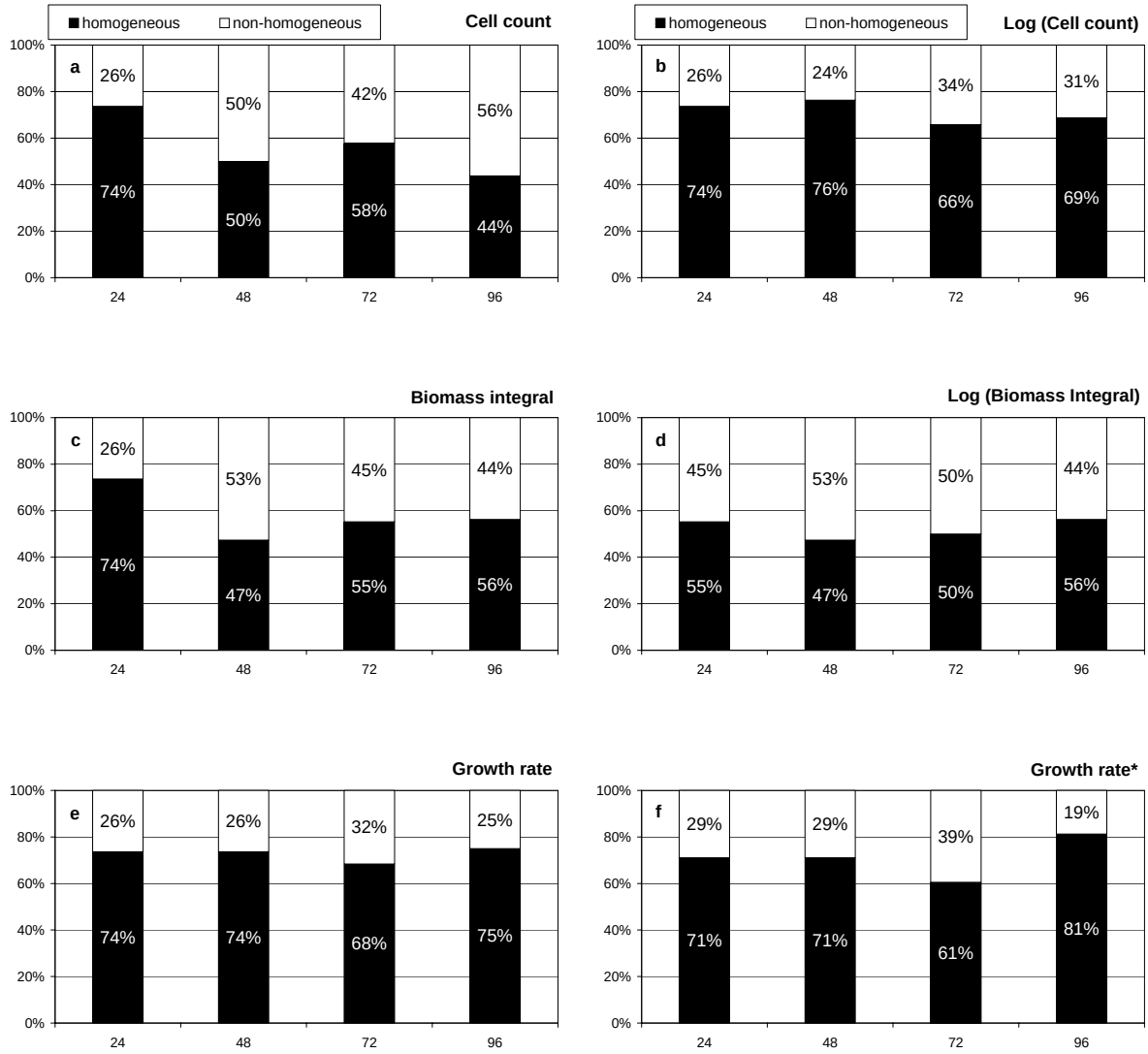
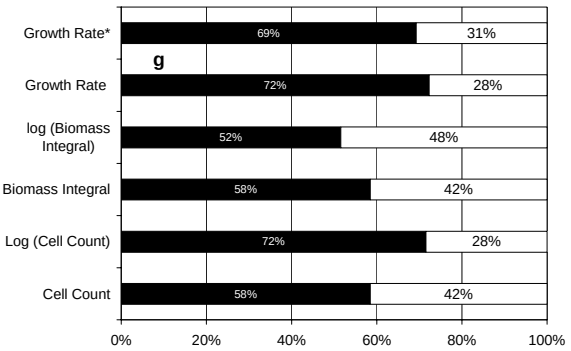


Figure A2: Results of Cochran's test, examining the variance homogeneity of the response variables "cell count" (a), "log (cell count)" (b), "biomass integral" (c), "log (biomass integral)" (d), "average growth rate" (e) and section by section growth rate ("Growth rate*)" (f); g: all samples lumped over time (α= 0.05; on average n = 130 per response variable)



A1.2 Prerequisites for dose/response curve modelling to derive an ECx

Dose/response curve modelling includes finding an appropriate mathematical function, which describes the dose/response relationship and which can be used to calculate values for any ECx (so-called "point estimates"). The most convenient way is to choose a model which can

be expressed as a linear function by some transformation of the response variable and/or concentration values, thus allowing curve fitting by a linear regression analysis. Although curve fitting can be performed directly using the non-transformed response variable values, commonly the toxicant caused deviation is expressed as response relative to the control, R, according to Eq. A1 or more frequently is transformed in %inhibition relative to the control after Eq. 4. These calculations require the arithmetic means of treatment and control replicates.

$$R = \frac{P_T}{P_C} \qquad \text{(A1)}$$

with:

P_T: arithmetic mean of the parameter (C, A, μ) in a treatment
P_C: arithmetic mean of the parameter (C, A, μ) in the control

The relative expressions of the toxicant-caused modification of a response variable's value allow better comparison of dose/response curves from different substances or conditions. In many cases – in particular if the concentration range was appropriately chosen -, these relative responses follow a sigmoid curve with increasing log (concentration) and thus can be described by the normal sigmoid, the logistic and the Weibull function, for which linear transformations are existing. A presentation and comparison of these linearisations is given in Christensen (1984). After such sort of transformation, a linear regression analysis can be performed.

Sokal & Rohlf (1981) summarise the prerequisites for a linear regression model of type I, applicable for the dose/response curve fitting considered here:

1. The independent variable X (i.e. the concentration of the test substance) is measured without error or is under control of the experimenter
2. The expected value for the variable Y for any given X is described by $\mu_Y = \alpha+\beta X$, i.e. the parametric means μ_Y of the values of Y are a function of X and lie on a straight line described by this equation.
3. For any given X_i of X the Y's are independently and normally distributed. This can be represented by the equation $Y_i = \alpha + \beta X_i + \varepsilon_i$, where ε_i's are assumed to be normally distributed error terms with a mean of zero.
4. The Y's along the regression line are assumed to be homoscedastic; that is, they have a common variance, σ^2, which is the variance of the ε_i's in the above expression. This means that the variance around the regression line is constant and hence is independent of the magnitude of X or Y.

In the present study, it could be shown that for every response variable the sample replicate values were normally distributed in more than 80% of the data sets. According to the Central Limit Theorem of Statistics, the normal distribution applies in particular also for the sample derivatives, such as the arithmetic means, even if the sample replicate values do not follow a normal distribution. According to Hartung (1984), the expected value for the quotient of two normally-distributed parameters, such as P_T and P_C of Eg. 4 and 7, is $E(f(X_1/X_2)) \approx \mu_1/\mu_2$ with the variance of Eq. A2.

$$\sigma^2_{X_1/X_2} = \frac{1}{\mu_2^2}\sqrt{\mu_1^2\sigma^2_{X_2} + \mu_2^2\sigma^2_{X_2}} \qquad \text{(A2)}$$

This theoretical consideration applies to the relative responses, R, as obtained by Eq. A1 and to the %inhibitions of Eq. 4. In order to examine, whether the normal distribution and variance homogeneity hypothesis can be assumed for the %inhibition relative to the control (Eq. 4), replicate values were generated, using every data pair which could be formed from the control and the replicates of the considered treatment. These %inhibition replicates were tested for normal distribution and variance homogeneity. Because at very low values of the proportion P_T/P_C (Eg. 4 and 7) deviations from the normal distribution were to be expected, testing was performed separately for each concentration level. The results clearly indicate that (1) the overall degree of normal distribution was high (Figure A3 g) and (2) that the proportion of normally distributed data sets decreased with increasing number (≈ value) of concentration. The correspondence with the normal distribution was higher in the biomass-related response variables, whilst the decrease in proportion of normally distributed data sets was more pronounced in the growth rates.

The high degree of normal distribution among the replicates of controls and treatments suggests that also the arithmetic means, used with the regression analyses, fulfil the "normal distribution" prerequisite. Although this is predicted by the Central Limit Theorem of Statistics, and thus must not be confirmed by empirical data, it appeared worth to demonstrate that also at smaller sample sizes, possibly interfering with the prediction, the normality hypothesis holds in most cases.

Considering the second (more important) prerequisite "variance homogeneity" along the regression line, Eq. A2 gives advice how to calculate the magnitude of the variance of the proportion PT/PC (Eq. 4 and Eq. A2) and even implies that the variances at the various test concentrations will be different. This can be demonstrated by theoretical calculations using Eq. A2, in which as an example μ2 was set to 100 and μ1 varied between 10 and 90, thus simulating different inhibitions in the parameter values by a theoretical toxicant. Three variances, 10, 20 and 30, which were constant for μ2 and all values of μ1, were investigated. Figure A4 a shows that, although the single variances for μ1 and μ2 were constant, the resulting variances for the quotient μ1/μ2 depend on the value of the quotient. Hence, non-homogeneity of variances in relative responses and %inhibitions is predicted by theory and obviously cannot be avoided.

Indeed, it can be demonstrated by the variances of the generated %inhibition data, as used for the normal distribution testing, that variances are to a great extent non-homogeneous. A comparison of Figures A2 g and A4 b reveals that the proportion of homogeneous data sets drastically decreased subsequent to the %inhibition transformation. Although this was demonstrated by the variances of replicate values, the same is true also for the variance of the sample means, which differs only by the factor $1/\sqrt{n}$.

The above considerations presuppose that an adequate mathematical function has been chosen for the dose/response curve. If this is not the case, an additional component of variance inconstancies along the regression line arises, which could be investigated by means of a residual analysis. This however is beyond the scope of the present study and was not performed. The demonstrated problems with non-homogeneous variances along the regression line do not allow performing simple "normal" linear regression analyses. Since long, this problem has been recognised and solutions have been developed how to use a modified regression approach. The variance inconstancy is compensated by weighting functions and the method of maximum likelihood regression, as described by Finney (1971, 1978) and Weber (1980). Weighting functions for Probit-, Logit and Weibull-analyses of quantitative responses, as given here, are based on the Poisson distribution and are published, e.g., in Christensen (1984).

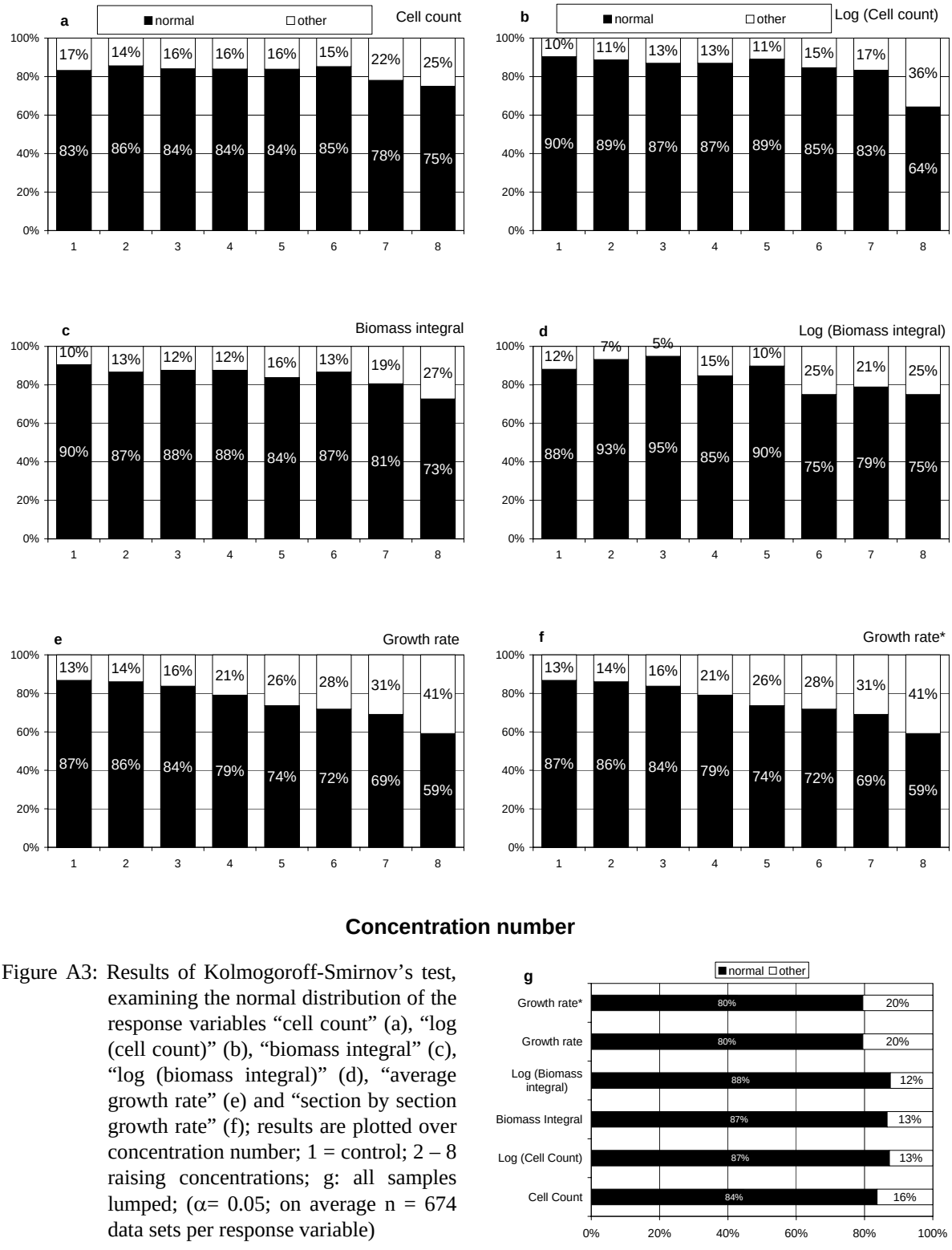


Figure A3: Results of Kolmogoroff-Smirnov's test, examining the normal distribution of the response variables "cell count" (a), "log (cell count)" (b), "biomass integral" (c), "log (biomass integral)" (d), "average growth rate" (e) and "section by section growth rate" (f); results are plotted over concentration number; 1 = control; 2 – 8 raising concentrations; g: all samples lumped; (α= 0.05; on average n = 674 data sets per response variable)

Summarising, the AGIT data sets under consideration exhibit properties which allow to compute the ECx by means of a probit, logit and Weibull maximum-likelihood regression analysis, provided these functions can be appropriately fitted to the data.

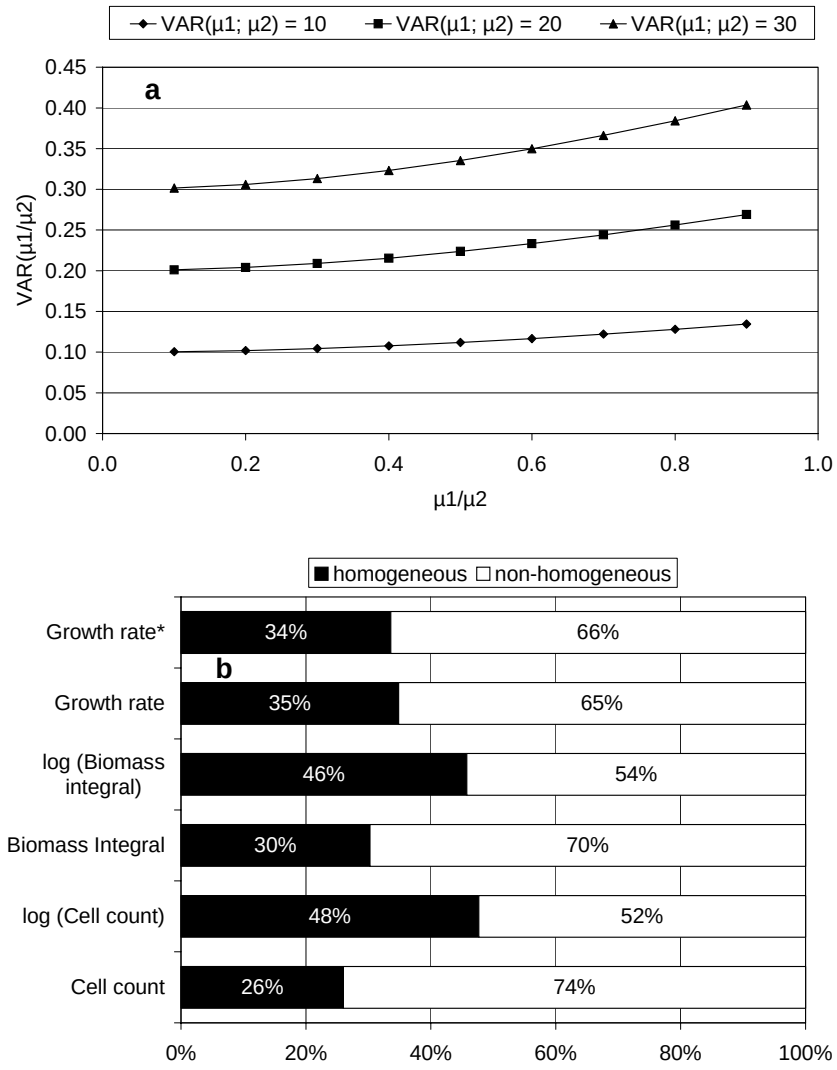


Figure A4: a) Theoretical calculation of the variance of the quotient µ1/µ2 according to Eq. A2; µ2 = 100; µ1 = 10, 20,:..,80, 90; b) Results of Bartlett's test, examining the variance homogeneity of the %inhibition in various response variables; all samples were lumped over time (α= 0.05; on average n = 108 per response variable)

A1.3 The behaviour of test parameters used or discussed as validity criterion

According to OECD, DIN and ISO guidelines (OECD 1984; DIN 1993; IS0 1989), a minimum growth rate should be maintained in the control, leading to an increase in cell density relative to the inoculated cell density by at least factor16 within 72 hours. The corresponding average growth rate is µ = 0.924. In the US standard guide for the AGIT (US EPA 1990), after 96 hours at least 10^5 cell per mL should be reached in the controls, which corresponds to an average growth rate µ = 0.58. Table 3 and 4 Figure A5 present control growth rates as observed for the various substances at different observation times. In addition, Figure A5 gives the above mentioned minimum average growth rates and the belt where the mean control growth rate is seen as optimal (DIN 1993). Less than one third of the control test cultures showed optimal average growth rates. Two tests do not fulfil the OECD/ISO/DIN validity criterion, but all of the tests meet the US-EPA criterion. The reasons for the on average sub-optimal growth rate in the controls cannot be identified at this point. Among them might be sub-optimal temperature and light conditions, bad performance of the algae culture, sub-optimal culture media, etc..

The coefficient of variation (CV) is being discussed as an additional validity criterion of the AGIT (Nusch, pers. comm.). The average CVs showed the following order from high to low values: “Biomass integral” (20.22%) > “Cell count” (16.81%), “Growth rate *” (16.65%) > “Growth rate” (11.56%) > “Log (Cell count)” (8.23%), Log (Biomass integral)” (7.74%). In all response variables the CVs tendentiously decreased during experimental time (Figure A6). High CVs at 24 to 48 h are obviously due to sample or counting errors at low cell densities.

In the currently used response variables, after 72 h the CVs were highest in „Cell count“ (16.24%), followed by „Biomass integral“ (15.71%) and „Growth rate“ (8.32%). The latter represents the average rate over time and thus has lower CVs than the section by section growth rate (“Growth rate*”) in which the values were similar to those in “Cell count”.

A2 Comparison of toxicity parameters

A2.1 Submitted results vs. those from the present study

Table A1 gives an overview over the results on the E_bC_{50} and E_rC_{50}, reported to the German Federal Environmental Agency, and those obtained by the present study. For the latter, only EC_{50}s were seen as valid if these did not exceed the 1.5-fold of the highest test concentration. Although in most cases corresponding results were obtained, in a substantial number of tests the differences in the EC_{50} proved to marked, obviously due to different methods used for the EC_{50} computation. The conclusion from this comparison thus is that the statistical methods need more standardisation.

A2.2 Statistical test, data transformation and the NOEC

It was shown that the log-transformation increased the degree of normal distribution and variance homogeneity in “Cell count”, whereas in “Biomass integral” the variance homogeneity was lower that in the non-transformed data. Hence this transformation cannot be recommended and only the log-transformation effect in “Cell count” will be investigated further. The purpose of this investigation is to examine whether the log-transformation leads to less sensitive (higher) NOECs or not. The log-transformation might lead to less sensitive NOECs as for example is the case in the growth rate. In addition, the NOECs from transformed data will be compared with those from the Bonferroni-U test – the non-parametric alternative test procedure. Results are given in Table A2.

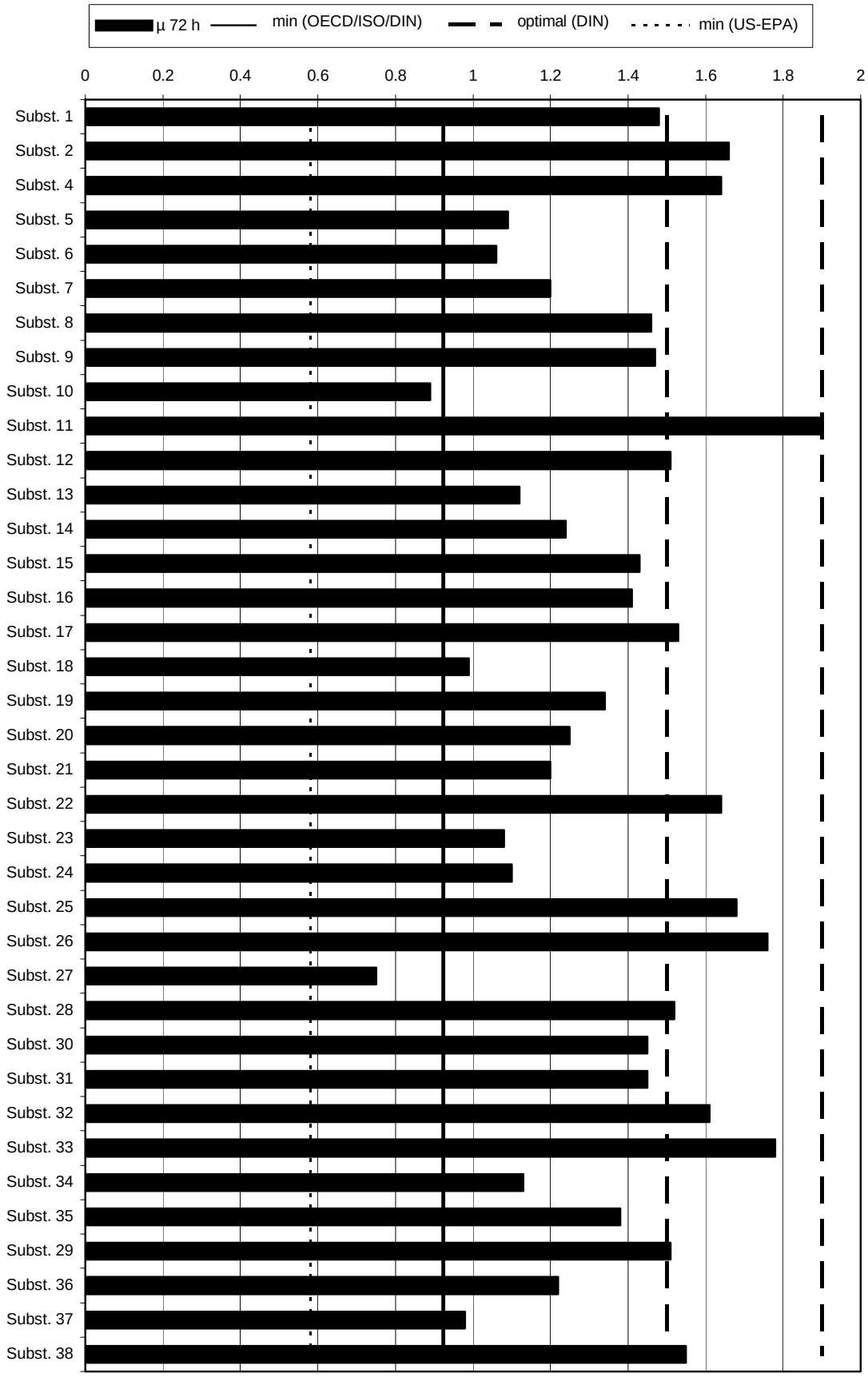


Figure A5: Average growth rate 0 – 72 h of control test cultures; min(OECD/ISO/DIN), min(US-EPA): minimum value prescribed as validity criterion by the respective guidelines; optimal (DIN): optimal range as given in DIN 1993

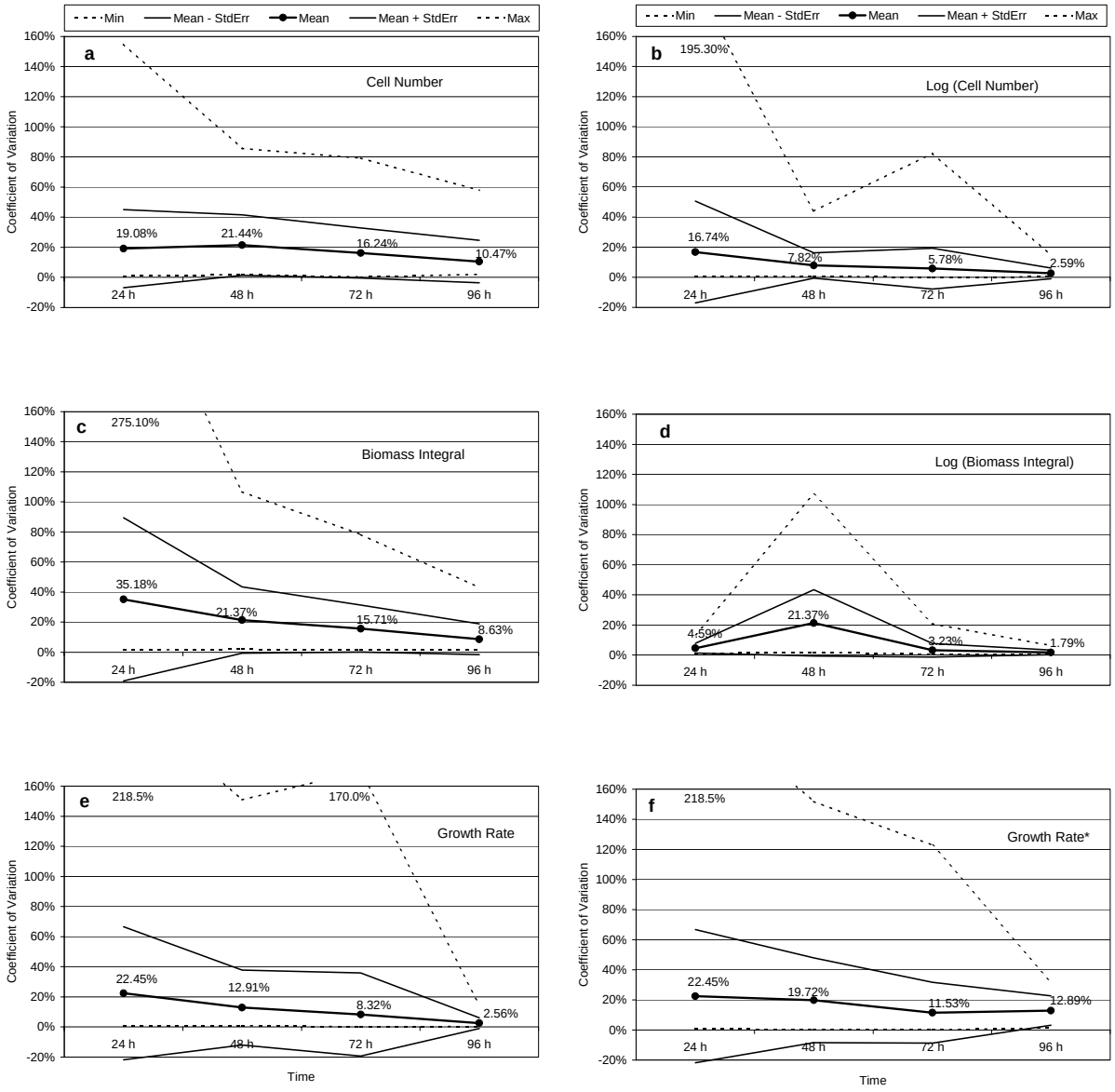


Figure A6: Mean coefficient of variation in control test cultures for the response variables “cell count” (a), “log (cell count)” (b), “biomass integral” (c), “log (biomass integral)” (d), “average growth rate” (e) and “section by section growth rate” (f); results are plotted over experimental time

Table A1: Overview over some results from submitted test reports as compared with those obtained in the present study

	Concentration	E_bC_{50} [mg/L]		E_rC_{50} [mg/L]	
Substance/Formulation	Range [mg/L]	Test Report	Study (72h)	Test Report	Study (72h)
Substance 1	1 - 100	n.s.	4.4	n.s.	n.d.
Substance 2	0.046 - 1.0	0.14	0.329	0.27	0.61[a]
Substance 4	0.063 - 2	n.s.	0.082 (48 h)	n.s.	2.76 (48 h).
Substance 5	1.6 - 400	n.s.	14.0	n.s.	82.9
Substance 6	0.039 - 40	2.4	3.43	17	17.8

Table A1: (continued)

Substance/Formulation	Concentration Range [mg/L]	E_bC_{50} [mg/L] Test Report	E_bC_{50} [mg/L] Study (72h)	E_rC_{50} [mg/L] Test Report	E_rC_{50} [mg/L] Study (72h)
Substance 7	10 - 100	26.7	21.6[a]	24.7	42.9[a]
Substance 8	0.0075 - 0.210	0.052/ 0.054	0.055	0.180/ 0.285	0.135
Substance 9	1 - 64	4863 [b]	n.d.	293 [b]	n.d.
Substance 10	0.0625 - 1.0	0.573	0.738	0.616 (24h)	0.727 (24 h)
Substance 11	5.6 - 100	34	41.9[m,a]	33	34.4 (48 h)
Substance 12	0.001 - 0.1	0.0013	0.0012	0.024	0.0225
Substance 13	0.1 - 22	0.47	0.7	1.2 (24-48h)	n.d.
Substance 14	0.24 - 5	1.6	1.88[a]	> 4.3	n.d.
Substance 15	0.158 - 500	2.99	1.78	352.5	267.4[a]
Substance 16	0.1 - 31.623	25.3	12.1	17.8 (24 -93h)	45.8
Substance 17	0.018 - 0.56	n.s.	0.069	n.s.	0.217
Substance 18	0.0125 - 0.8	0.22	0.105	0.8 (24-48h)	n.d.
Substance 19	6.25 - 100	37	39.5[m,a]	46	42.01[m,a]
Substance 20	0.01 - 100	n.s.	37.15a	n.s.	26.37a
Substance 21	0.01 - 100	n.s.	55.21[m,a]	n.s.	34.94[m,a]
Substance 22 (72h)	0.025 - 6.4	n.s.	0.218	n.s.	1.54
Substance 22 (120h)	0.025 – 6.4	0.054	0.24	0.6	2.30
Substance 23 (72h)	0.39 - 50	3.29/ 2.94	3.65[a]	7.69/ 50.94	4.7
Substance 23 (96h)	0.39 - 50	3.29/ 2.94	3.38	7.69/ 50.94	7.29
Substance 24 (72h)	0.054 - 1.5	1.34/ 1.69	1.74[a]	1.50/ 1.50	197
Substance 24 (120h)	0.054 - 1.5	1.34/ 1.69	1.33	1.50/ 1.50	128[c]
Substance 25	0.1 - 1.6	n.s.	0.1	n.s.	0.2[a]
Substance 26	1 - 56	n.s.	2.2[a]	n.s.	12.1[a]
Substance 27 (72h)	0.01 - 0.16	0.012	n.d.	0.015 (24-48h)	0.0078[c]
Substance 27 (120h)	0.01 - 0.16	0.014	n.d.	0.015 (24-48h)	0.012[m,c]
Substance 28	0.32 – 320	n.s.	45.6	n.s.	n.d.
Substance 30 Test 1	0.191 - 12.516	2.848	4.33[a]	291	n.d.
Substance 31 Test 2	0.013 - 0.402	n.s.	0.085	n.s.	0.213
Substance 32	0.18 - 3.2	n.s.	n.d.	n.s.	n.d.
Substance 34 (72h)	0.30 – 30	1.25	1.1	21.93	14.4[a]
Substance 34 (96h)	0.30 – 30	1.25	3.03	21.93	n.d.
Substance 33 (72h)	3.125 – 50	12.5	11.9	8 (0-24h)	29.8
Substance 33 (96h)	3.125 – 50	15	14.8	8 (0-24h)	62.6[c]
Substance 35	0.47 – 190	3.5	4.15	n.s.	n.d.
Substance 29	0.36 – 1200	4.8	4.25	160	176.9
Substance 36 (72h)	0.003 - 1.0	0.083/ 0.040	0.21[c]	0.037/ 0.031	n.d.
Substance 36 (96h)	0.003 - 1.0	0.083/ 0.040	0.073[a]	0.037/ 0.031	1.17[a]
Substance 37 (70h)	0.003 - 1.0	0.081	0.079	0.0343	0.633
Substance 37 (94h)	0.003 - 1.0	0.035	0.0324	0.019	0.173
Substance 38	0.012 - 12.0	0.78	0.654	11.96	15.7

a : Confidence limits not defined; b: Values do not appear logical; c: Upper confidence limit very large; m: EC_{50} was determined by moving averages

Table A2: Comparison the log-transformation in "Cell count" with non-transformed data. Given is the percentages of cases in which the NOEC from transformed data (NOEC*) was lower, equal or higher than that from non-transformed data in Dunnett's test (D/D), Williams test (W/W), and Bonferroni-U test (B/D, B/W).

	D/D	**W/W**	**B/D**	**B/W**
NOEC* lower	2.5%	4.1%	31.8%	40.9%
NOEC* equal	82.2%	80.5%	63.6%	59.1%
NOEC* higher	15.3%	15.4%	4.5%	0.0%
Number of cases	118	123	22	22

At least for the selection of AGITs, as investigated by the present study, for the Dunnett's and Williams test, it was found that in a substantial number of cases (about 15%) the NOECs were higher after transformation, thus less sensitive. On the other hand, the NOECs from transformed data were in more than 30% of cases, where NOECs from the Bonferroni-U test could be obtained, lower than in this test. In addition in about 80% of cases, no NOEC could be determined by the Bonferroni-U test. Therefore, if necessary, the transformation should be preferred.

As was expected from the different power of the statistical tests (Williams > Dunnett > Bonferroni-U), in many cases the NOECs was lowest, if the most powerful test was applied (Table A3) In 17% (Biomass integral) to 28% of tests the Williams test revealed lower (more sensitive) NOECs than the remaining two statistical tests. Even when the Williams test appeared less sensitive than the Dunnett's test, a closer examination shows that the Williams NOEC was more reasonable as is exemplified by Table A4.

Table A3: Comparison of the NOECs obtained from the Dunnett's test (NOEC D), the Williams test (NOEC W) and the non-parametric Bonferroni-U test (NOEC B)

	Cell Count	**Log (Cell count)**	**Biomass Integral**	**Growth Rate**	**Growth Rate***
NOEC W > NOEC D	2%	2%	2%	2%	4%
NOEC W = NOEC D	74%	77%	80%	75%	69%
NOEC W < NOEC D	25%	21%	17%	24%	28%
Number of cases	121	128	127	123	112
NOEC B > NOEC D	54%	27%	48%	19%	12%
NOEC B = NOEC D	46%	68%	52%	81%	88%
NOEC B < NOEC D	0%	5%	0%	0%	0%
Number of cases	24	22	21	21	17

With Substance 24 (Table A4a) the dose response curve did not monotonously decrease with increasing concentration. In other words, Dunnett's test indicated alternatively significant and non-significant deviations. In contrast in the Williams test procedure, the maximum likelihood course of the dose/response curve is used with testing, thus smoothing the irregularities in the original data, so that switching between significance and non- significance cannot occur anymore, thus leading to a more likely NOEC. In the example of Substance 8 (Table A4 b) Dunnett's test tracked down one single response as significant in the middle of the concentration range. The maximum-likelihood smoothing in the Williams test revealed no significant difference at all, which obviously is reasonable in this example. Summarising, using the Williams test prevents non-reasonable NOEC in non-monotonous dose/response relationships and leads to lower (more sensitive) NOECs in (more) monotonous relationships.

In the Bonferroni-U test only in 17% of cases NOECs could be determined. In the remaining cases none of the treatments showed a significant difference to the control (NOEC > highest test concentration). From the tests, for which the Bonferroni-U test delivered results, 12-54 % revealed higher NOECs than Dunnett's test (implicitly also Williams test) and 46-88% the same NOEC. Only in one case (5%) the NOEC B was lower than the NOEC D. The reason was that due to a high variance used with Dunnett's test, the NOEC-B concentration was assessed to be non-significant.

Fortunately, the degree of normal distribution and variance homogeneity appears to be high in the AGIT, so that parametric tests normally can (and should) be performed. From these the Williams test should be preferred due to it higher statistical power and its ability to exert some smoothing of the dose response relationship, so that more reasonable NOECs will be obtained.

Table A4: Examples of Substance 24 (a, Cell count) and Substance 8 (b, Growth rate*), in which Williams test produced a higher or no NOEC as compared with Dunnett's test. X: arithmetic mean; s: standard deviation; n: number of replicates; t: sample t; t_d, t_w: tabelled t (α = 0.05, one-sided smaller); mlX: maximum likelihood estimate; +,-: significant, non-significant. Note that the Williams test is performed using the maximum likelihood estimates rather than the original values; for further explanation see text.

a

				Dunnett's test			Williams test			
mg/L	X	S	N	T	t_d		mlX	T	t_w	
S.C	5.667	1.1273	3							
0.054	7.250	1.7500	3	1.63	-2.56	-	6.458	0.817	-1.746	-
0.130	6.250	1.0000	3	0.60	-2.56	-	7.694	2.092	-1.831	-
0.230	9.083	1.6073	3	3.53	-2.56	-	7.694	2.092	-1.860	-
0.370	7.750	1.3229	3	2.15	-2.56	-	7.694	2.092	-1.873	-
0.790	2.333	0.3819	3	-3.44	-2.56	+	3.917	-1.806	-1.882	-
1.100	5.500	1.1456	3	-0.17	-2.56	-	3.917	-1.806	-1.887	-
1.500	2.083	0.3819	3	-3.70	-2.56	+	2.083	-3.697	-1.890	+

b

				Dunnett's test			Williams test			
mg/L	X	S	n	T	t_d		mlX	t	t_w	
Control	1.492	0.1878	3							
0.008	1.649	0.4078	3	0.51	-2.53	-	1.571	0.254	-1.761	-
0.015	0.745	0.2975	3	-2.42	-2.53	-	0.928	-1.833	-1.849	-
0.028	0.519	0.2421	3	-3.16	-2.53	+	0.928	-1.833	-1.878	-
0.057	0.919	0.2952	3	-1.86	-2.53	-	0.928	-1.833	-1.892	-
0.11	1.381	0.3639	3	-0.36	-2.53	-	0.928	-1.833	-1.901	-
0.21	1.073	0.6548	3	-1.36	-2.53	-	0.928	-1.833	-1.906	-

A2.3 Comparison of EC_{10}, EC_{20} and EC_{50} with the NOEC

According to OECD (1998) the NOEC will be phased out in near future and will be replaced by some ECx, where x is small. In order to provide background information on which of the ECx might be appropriate for replacement of the NOEC the ratio ECx/NOEC was investigated for the response variables. For the considered response variables values of the EC_{10}, EC_{20}, EC_{50} and NOEC at different experimental times are presented in Tables A6 to A9. Only those values of the ECx were considered for the ratio calculations which ranged from the 0.1-fold of the lowest to the 1.5-fold of the highest tested concentration. In other words, wider extrapolated ECx-values were not included. Both the NOECs from Dunnett's test (NOEC D) and from Williams test (NOEC W) were used, in case the prerequisites were fulfilled. If this did not apply, NOECs from the Bonferroni U-Test (NOEC B) were inserted. However, as

shown above, the latter could not be always determined, so that also for these cases the NOEC D or NOEC W was used as a first approximation (in Table A6 to A9 these cases are marked). The general conclusions are supposed to be unaffected by this. Generally, cases in which the NOEC was zero or higher than the highest test concentration were not considered.

Figure A7 gives cumulative distributions of the logarithm of the ECx/NOEC ratio, showing a good correspondence to normal distributed. In Table A5, the percentages of cases are given in which the ratio was < 1 and >= 1. The ratio 1 (log (ratio) = 0) is indicated by a vertical line. Depending on the response variable, 3.8% (Growth rate) to 10% (Cell count) of the NOECs D were higher than the EC_{50}. In the NOEC W this range was 2.5% to 11%. As already demonstrated above, the higher statistical power of the Williams test led to lower values of the NOEC, thus higher values of the ratio and a slight shift of the curves to the right.

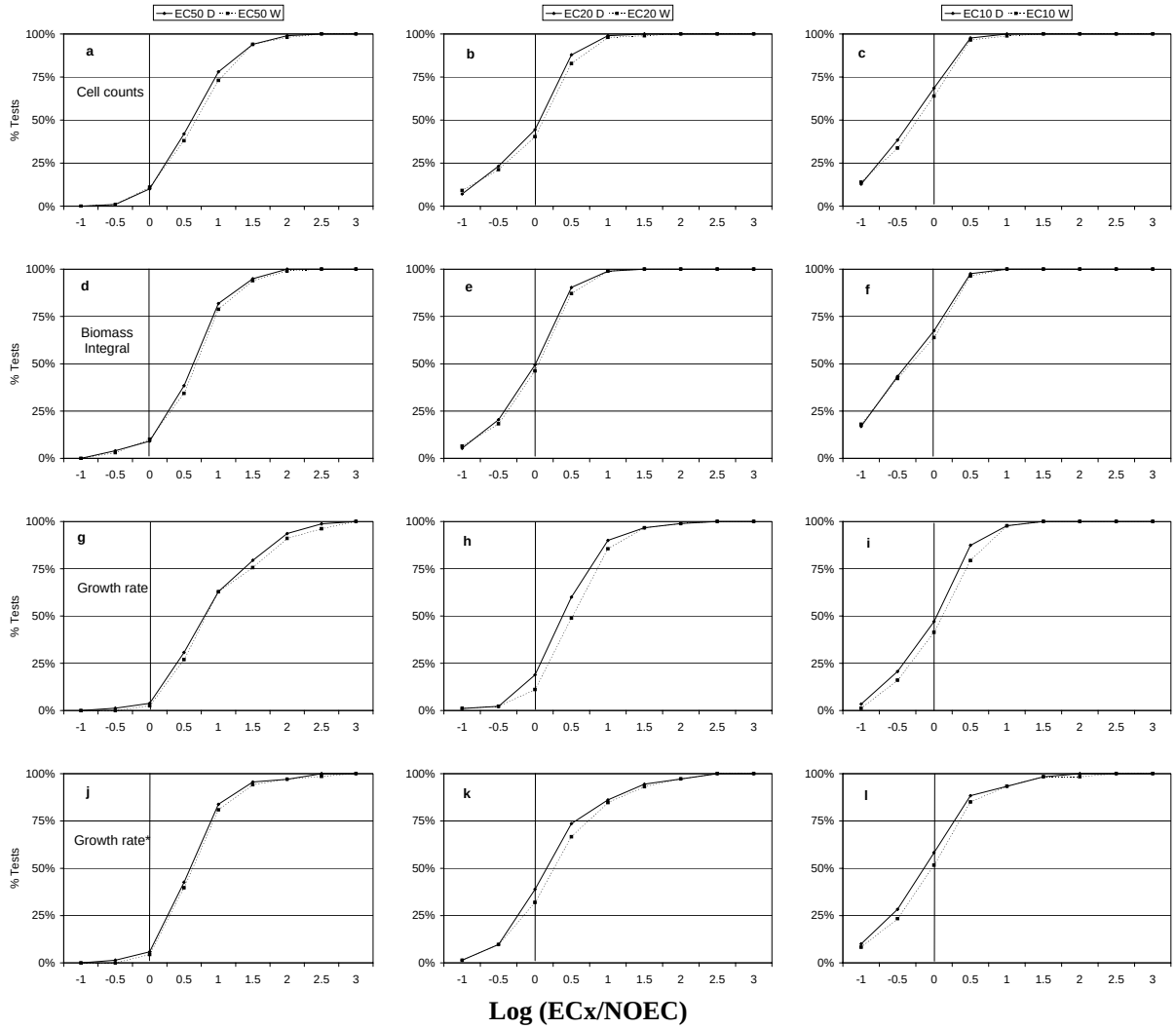


Figure A7: Distribution of the ECx/NOEC for “Cell count” (a-c), “Biomass integral” (d-f), “Growth rate” (g-i) and “Growth rate*” (j-l). Vertical lines indicate the location where ECx = NOEC. Left from the vertical line the NOEC was greater than the ECx. Solid lines: NOEC from Dunnett’s test; broken lines: NOEC from Williams test.

Table A5: Percentages where the ratio ECx/NOEC was greater equal or smaller than the ECx (see vertical lines in Figure A7); for explanation see text

		EC_{50}/ NOEC D	**EC_{50}/ NOEC W**	**EC_{20}/ NOEC D**	**EC_{20}/ NOEC W**	**EC_{10}/ NOEC D**	**EC_{10}/ NOEC W**
Cell count	>=1	90.0%	89.0%	55.6%	59.6%	31.4%	36.0%
	<1	10.0%	11.0%	44.4%	40.4%	68.6%	64.0%
Biomass integral	>=1	90.9%	89.9%	50.5%	53.8%	32.5%	36.1%
	<1	9.1%	10.1%	49.5%	46.2%	67.5%	63.9%
Growth rate	>=1	96.3%	97.5%	81.1%	88.9%	52.9%	58.6%
	<1	3.8%	2.5%	18.9%	11.1%	47.1%	41.4%
Growth rate*	>=1	94.2%	95.7%	61.1%	68.1%	41.7%	48.3%
	<1	5.8%	4.3%	38.9%	31.9%	58.3%	51.7%

About 19 to 49% of the NOEC D and 11 to 46% of the NOEC W were smaller than the EC_{20}, and 58 to 69% of the NOEC D and 52 to 64% of the NOEC W were smaller than the EC_{10}. In other words, in "Cell count" and "Biomass integral" the NOEC corresponded nearly the EC_{20}, in the section by section growth rate (Growth rate *) some value between EC_{20} and EC_{10}, and in the "Growth rate" the EC_{10}.

For the final conclusions one has to consider, that the NOEC is a rather conservative estimate of the NEC at higher variances and low replication, since significant results (LOECs) are obtained at concentration exerting already a substantial effect. Hence the NOEC should not be used for calibration any ECx. This is especially true at higher variances and low replication. Therefore, it would be no improvement if in the "Cell count" and "Biomass integral" the NOEC would be replaced by the EC_{20}. In contrast, in the growth rates with lower variances the NOEC might be replaced by the EC_{10}. If a decision like that would be made, only those tests should be accepted in which a clear dose/response relationship is to be observed and thus a function can be fitted from which an ECx (x = small) can be derived.

Table A6: Toxicity parameter values for "Cell Count"; NOEC D: NOEC from Dunnett's test; NOEC W: NOEC from Williams Test; NOEC B: NOEC from Bonferroni-U test; values marked with an asterisk indicate lacking prerequisites for the statistical test, rest see text

	EC_{10}	EC_{20}	EC_{50}	NOEC D	NOEC W	NOEC B
24 h						
Substance 1	0.1	1.4		32	1	
Substance 2	0.017	0.048	0.341	0.046	0.046	
Substance 3	0.155	0.249	0.62	0.21	0.21	
Substance 4	0.021	0.053	0.299	0.5	0.5	
Substance 5		0.16		1.6	1.6	
Substance 6	0.019	0.178	13.463			
Substance 7			88.71	31.6	31.6	
Substance 8	0.014	0.029	0.117	0.057	0.057	
Substance 9		0.9		8	8	
Substance 10	0.1046	0.3218		0.0625	0.0625	
Substance 11			47	32	32	
Substance 12				0.001*	0.001*	
Substance 13	0.02	0.11	3.43	0.1	0.1	
Substance 14	0.52	1.72		1.1	0.5	
Substance 15	0.283	1.94	77.225			5
Substance 16	1.085	0.354			1*	
Substance 17	0.01	0.027	0.184	0.032	0.018	

Table A6 (continued)

	EC_{10}	EC_{20}	EC_{50}	NOEC D	NOEC W	NOEC B
Substance 18		0.009	0.759	0.025*	0.025*	
Substance 19	19.32	25.89	45.36	6.25	6.25	
Substance 20				10*	10*	
Substance 21			130.03	1	1	
Substance 22	0.024	0.135	3.62	0.1	0.05	
Substance 23	2.48	3.36	5.97	0.78	0.78	
Substance 24				-	-	
Substance 25			0.3	-	-	
Substance 26			24.2	1	1	
Substance 27	0.0042	0.0086	0.0331	0.01	0.01	
Substance 28	21.25	129.79		32	32	
Substance 29	0.07	7.87				0.36
Substance 30				-	-	
Substance 31	0.024	0.044	0.139	0.025	0.025	
Substance 32				0.32	0.18	
Substance 33	1.663	2.969	8.999	3.125*	3.125*	
Substance 34			2.09	0.95	0.3	
Substance 35	0.17	0.9	21.37	17	17	
Substance 36	0.704				0.316*	
Substance 37					0.003	
Substance 38	0.024	0.103	1.688			3.8
48 h						
Substance 1	0.5	1.5	12.7	3.2	1	
Substance 2	0.103	0.157	0.352	0.1	0.1	
Substance 3	0.107	0.162	0.36	0.1	0.1	
Substance 4						
Substance 5	0.42	6.07		25*	6.25*	
Substance 6	0.255	0.871	9.11			
Substance 7		1.21	6.3			17.7
Substance 8	0.001	0.003	0.03	0.0075	0.0075	
Substance 9		48.8		8*	8*	
Substance 10	0.238	0.4331	1.3622	0.125	0.125	
Substance 11			45.5	32	32	
Substance 12		0.0001	0.0009	0.001*	0.001*	
Substance 13	0.01	0.03	0.43	0.1*	0.1*	
Substance 14		0.09	1.93	0.5	0.5	
Substance 15	0.025	0.157	5.355	0.158	0.158	
Substance 16					0.1	
Substance 17	0.014	0.023	0.055	0.032	0.032	
Substance 18		0.006	0.097	0.0125	0.0125	
Substance 19	29.56	32.37	38.53	25*	25*	
Substance 20			66.64	10*	10*	
Substance 21	2.37	4.41	14.38	1*	1*	
Substance 22	0.018	0.046	0.284	0.025*	0.025*	
Substance 23	2.34	2.84	4.13	1.56*	1.56*	
Substance 24		0.012		0.37	1.1	
Substance 25			0.1	0.8*	0.1*	
Substance 26	0.1	0.3	1.7	1*	5.6*	
Substance 27	0.0084	0.0109	0.018	0.01*	0.01*	
Substance 28	4.54	12.57	88.27	10*	3.2*	
Substance 29	0.08	0.44	10.25	0.36	0.36	
Substance 30	0.039	0.283	12.583	1.018*	0.716*	
Substance 31	0.034	0.046	0.083	0.025	0.025	
Substance 32	0.06	0.76		0.32	0.32	

Table A6 (continued)

	EC_{10}	EC_{20}	EC_{50}	NOEC D	NOEC W	NOEC B
Substance 33	2.216	4.054	12.877	3.125	3.125	
Substance 34				0.95	0.3	
Substance 35	0.06	0.27	4.45	1.7*	0.47*	
Substance 36	0.003	0.014	0.315			0.01
Substance 37		0.0003	0.0739			0.003
Substance 38	0.049	0.131	0.852	0.12	0.12	
72 h						
Substance 1			1.7	1	1	
Substance 2	0.106	0.152	0.303	0.1	0.1	
Substance 3	0.106	0.152	0.303	0.1	0.1	
Substance 4				1	1	
Substance 5	0.61	1.59	9.91	1.6*	1.6*	
Substance 6	0.401	0.859	3.695	10*	10*	
Substance 7	2.75	4.85	14.39			17.7
Substance 8	0.025	0.036	0.069	0.015	0.0075	
Substance 9		35.9	4.9	8	8	
Substance 10	0.2292	0.3376	0.7081	0.125	0.125	
Substance 11	32.7	35.3	41	32	18	
Substance 12	0.0001	0.0003	0.0018	0.001*	0.001*	
Substance 13	0.09	0.23	1.47	0.22*	0.1*	
Substance 14			1.72	0.5	0.5	
Substance 15	0.07	0.225	2.095			0.158
Substance 16	1.374	3.781	26.261	10	0.1	
Substance 17	0.026	0.038	0.079	0.1	0.056	
Substance 18		0.007	0.28	0.0125	0.0125	
Substance 19			41.93	25	25	
Substance 20			33.83	10*	10*	
Substance 21			114.55	10*	10*	
Substance 22	0.038	0.067	0.2	0.05*	0.05*	
Substance 23	2.04	2.51	3.73	1.56*	0.78*	
Substance 24	0.073	0.333		0.23	0.13	
Substance 25		0.1	0.1	0.1*	0.1*	
Substance 26	0.2	0.5	2.6	1*	5.6*	
Substance 27	0.0092	0.0114	0.0172	0.01*	0.01*	
Substance 28	1.52	4.43	34.47	1	1	
Substance 29	0.12	0.39	3.43	0.36	0.36	
Substance 30		0.04	3.011	0.411	0.411	
Substance 31	0.04	0.053	0.091	0.025	0.025	
Substance 32	1.57			-	-	
Substance 33	5.608	8.045	16.046	3.125*	3.125*	
Substance 34		0.33		0.3	0.3	
Substance 35	0.11	0.43	5.59	0.47	0.47	
Substance 36	0.003	0.008	0.057			0.032
Substance 37	0.0006	0.0025	0.04	0.003	0.003	
Substance 38	0.036	0.099	0.672			0.38
Substance 1			0.6	1	1	
Substance 4	0.482			1	0.5	
Substance 5	0.94	1.79	6.15	1.6	1.6	
Substance 8	0.018	0.026	0.05	0.015	0.0075	
Substance 10	0.2325	0.3205	0.5924	0.125	0.125	
Substance 12	0.003	0.0038	0.0061	0.0032*	0.0032*	
Substance 16	0.342	0.825	4.443	0.1	10	
Substance 19			39.08	25	25	
Substance 22	0.051	0.084	0.216	0.1*	0.05*	

Table A6 (continued)

	EC_{10}	EC_{20}	EC_{50}	NOEC D	NOEC W	NOEC B
Substance 23	1.02	1.52	3.23	0.39*	0.39*	
Substance 24	0.054	0.137	0.816	0.13*	0.054*	
Substance 27	0.0107	0.0125	0.0171	0.01*	0.01*	
Substance 33	5.338	8.453	20.372	6.25*	6.25*	
Substance 34	1	2.5	14.62	0.95	0.95	
Substance 36	0.004	0.009	0.034			0.032
Substance 37	0.0008	0.0022	0.0151			0.003

Table A7: Toxicity parameters for “Biomass Integral”; NOEC D: NOEC from Dunnett’s test; NOEC W: NOEC from Williams Test; NOEC B: NOEC from Bonferroni-U test; values marked with an asterisk indicate lacking prerequisites for the statistical test, rest see text

	EC_{10}	EC_{20}	EC_{50}	NOEC D	NOEC W	NOEC B
24 h						
Substance 1		0.5	106.1	32	1	
Substance 2	0.017	0.04	0.203	0.046	0.046	
Substance 3	0.146	0.22	0.483	0.21	0.21	
Substance 4				0.5	0.5	
Substance 5			82.04	1.6	1.6	
Substance 6	0.019	0.086	1.505		10	
Substance 7			92.62	56.2	56.2	
Substance 8	0.005	0.011	0.049	0.057	0.057	
Substance 9				8	8	
Substance 10	0.1142	0.2001	0.5858	0.0625	0.0625	
Substance 11			40.8	32	32	
Substance 12				0.001*	0.001*	
Substance 13	0.02	0.08	1.49	0.1	0.1	
Substance 14	0.4	1.15		1.1	0.5	
Substance 15	0.123	0.925	43.665			5
Substance 16	1.613	0.635	0.107	1*	1*	
Substance 17	0.011	0.023	0.097	0.032	0.018	
Substance 18	0.002	0.007	0.044	0.025*	0.025*	
Substance 19	22.66	26.02	33.93	6.25	6.25	
Substance 20			108.9	10*	10*	
Substance 21			3.49	10*	1*	
Substance 22	0.019	0.082	1.326	0.1	0.05	
Substance 23	1.52	2.05	3.64	0.78	0.39	
Substance 24						
Substance 25						
Substance 26				1	1	
Substance 27				0.01	0.01	
Substance 28	8.01	31.64	438.67	32	32	
Substance 29		0.09				0.36
Substance 30						
Substance 31	0.027	0.04	0.088	0.025	0.025	
Substance 32		4.29		0.32	0.18	
Substance 33	3.015	3.723	5.573	3.125*	3.125*	
Substance 34				0.3	0.3	
Substance 35				17	17	
Substance 36	0.521			0.316*	0.316*	
Substance 37				0.003	0.003	
Substance 38	0.122	0.19	0.447	0.38	0.38	

Table A7 (continued)

	EC_{10}	EC_{20}	EC_{50}	NOEC D	NOEC W	NOEC B
48 h						
Substance 1	0.3	1.1	14.7	1	1	
Substance 2	0.073	0.12	0.309	0.1	0.1	
Substance 3	0.113	0.17	0.373	0.1	0.1	
Substance 4			0.082	0.5	0.25	
Substance 5		1.24	119.06	1.6	1.6	
Substance 6	0.07	0.254	2.995	10*	10*	
Substance 7		2.27	12.59			31.6
Substance 8	0.009	0.016	0.05	0.028	0.0075	
Substance 9	0.1	20.1		8*	8*	
Substance 10	0.1684	0.2892	0.8141	0.125	0.0625	
Substance 11			44	32	32	
Substance 12			0.0006	0.001*	0.001*	
Substance 13	0.01	0.04	0.53	0.1	0.1	
Substance 14	0.08	0.27	3.05	1.1	0.5	
Substance 15	0.021	0.163	8.59	0.158	0.158	
Substance 16				1*	1*	
Substance 17	0.018	0.027	0.061	0.032	0.032	
Substance 18	0.003	0.007	0.055	0.0125*	0.0125*	
Substance 19	27.79	30.25	35.6	6.25*	6.25*	
Substance 20			56.19	10*	10*	
Substance 21	2.08	3.64	10.68	1*	1*	
Substance 22	0.017	0.048	0.347	0.025*	0.025*	
Substance 23	2.43	2.81	3.73	1.56*	1.56*	
Substance 24	0.764	0.925	1.334	1.1	1.1	
Substance 25				0.4*	0.1*	
Substance 26	0.1	0.2	1.8	1	5.6	
Substance 27				0.01*	0.01*	
Substance 28	4.52	12.61	89.67	10*	10*	
Substance 29	0.05	0.34	12.18	0.36	0.36	
Substance 30	0.052	0.294	8.019	1.018*	0.716*	
Substance 31	0.033	0.045	0.08	0.025	0.025	
Substance 32	0.04	0.78		0.32	0.18	
Substance 33	2.265	3.485	7.949	3.125*	3.125*	
Substance 34				0.3	0.3	
Substance 35	0.14	0.39	2.85	0.47*	0.47*	
Substance 36	0.007	0.055				0.032
Substance 37		0.0041				0.01
Substance 38	0.073	0.15	0.591	0.12	0.12	
72 h						
Substance 1		0.1	4.4	1	1	
Substance 2	0.11	0.158	0.317	0.1	0.1	
Substance 3	0.118	0.168	0.329	0.1	0.1	
Substance 4				1	1	
Substance 5	0.44	1.44	13.97	1.6	1.6	
Substance 6	0.256	0.623	3.425	10*	10*	
Substance 7	5.14	8.41	21.61			17.7
Substance 8	0.013	0.021	0.055	0.0075*	0.0075*	
Substance 9	0.1	70		8	8	
Substance 10	0.2098	0.3231	0.738	0.125	0.125	
Substance 11			41.9	32	32	
Substance 12		0.0001	0.0012	0.001*	0.001*	
Substance 13	0.03	0.08	0.7	0.1*	0.1*	

Table A7 (continued)

	EC_{10}	EC_{20}	EC_{50}	NOEC D	NOEC W	NOEC B
Substance 14		0.08	1.88	0.5	0.5	
Substance 15	0.021	0.098	1.781	0.158	0.158	
Substance 16				10	10	
Substance 17	0.02	0.03	0.069	0.056	0.056	
Substance 18		0.006	0.105	0.0125*	0.0125*	
Substance 19			39.52	25	25	
Substance 20			37.15	10*	10*	
Substance 21			55.21	10*	10*	
Substance 22	0.032	0.061	0.218	0.05*	0.025*	
Substance 23	2.3	2.7	3.65	1.56*	1.56*	
Substance 24	0.407	0.67	1.737	0.37	0.37	
Substance 25		0.1	0.1	0.1*	0.1*	
Substance 26	0.2	0.4	2.2	1*	5.6*	
Substance 27				0.01*	0.01*	
Substance 28	2.27	6.36	45.63	1*	1*	
Substance 29	0.1	0.37	4.25	0.36	0.36	
Substance 30	0.108	0.383	4.332	0.411	0.411	
Substance 31	0.038	0.05	0.085	0.025	0.025	
Substance 32	0.03			0.32	1.8	
Substance 33	3.335	5.162	11.914	3.125	3.125	
Substance 34				0.3	0.3	
Substance 35	0.12	0.4	4.15	0.47*	0.47*	
Substance 36	0.002	0.01	0.21			0.032
Substance 37		0.0015	0.0794	0.003	0.003	
Substance 38	0.044	0.112	0.654			0.38
96 h						
Substance 1			1.5	1	1	
Substance 4	0.845	2.46		0.5	0.5	
Substance 5	0.87	1.75	6.6	1.6	1.6	
Substance 8	0.016	0.025	0.055	0.0075	0.0075	
Substance 10	0.224	0.3205	0.6365	0.125	0.125	
Substance 12	0.0008	0.0014	0.0041	0.001*	0.001*	
Substance 16	0.36	1.204	12.128	10	10	
Substance 19			39.44	25	25	
Substance 22	0.045	0.076	0.212	0.05*	0.025*	
Substance 23	1.5	1.98	3.38	0.78*	0.78*	
Substance 24	0.158	0.32	1.233	0.13	0.13	
Substance 27				0.01*	0.01*	
Substance 33	4.136	6.399	14.752	3.125	3.125	
Substance 34	0.11	0.35	3.03			0.3
Substance 36	0.002	0.007	0.073			0.032
Substance 37	0.0003	0.0016	0.0324			0.003

Table A8: Toxicity parameters for "Growth Rate"; NOEC D: NOEC from Dunnett's test; NOEC W: NOEC from Williams Test; NOEC B: NOEC from Bonferroni-U test; values marked with an asterisk indicate lacking prerequisites for the statistical test, rest see text

	EC_{10}	EC_{20}	EC_{50}	NOEC D	NOEC W	NOEC B
24 h						
Substance 1	0.5	10.4		32	1	
Substance 2	0.039	0.099	0.591	0.046	0.046	
Substance 3	0.247	0.366	0.776	0.21	0.21	
Substance 4				1*	1*	
Substance 5				1.6	1.6	
Substance 6	0.041	0.218	5.38	0.156	0.039	
Substance 7			97.57	56.2	56.2	
Substance 8	0.013	0.026	0.095	0.057	0.057	
Substance 9		0.1		8*	8*	
Substance 10	0.1975	0.3089	0.7271	0.0625	0.0625	
Substance 11			31.6	32*	32*	
Substance 12		0.0001	0.0036	0.001	0.001	
Substance 13	0.23	0.82	9.4	0.46	0.1	
Substance 14	0.31	5.58		1.1	1.1	
Substance 15		0.229	56.058	0.5	0.158	
Substance 16	0.853	0.312		0.1	0.1	
Substance 17	0.021	0.046	0.205	0.056	0.032	
Substance 18	0.005	0.014	0.087	0.025*	0.025*	
Substance 19	29.68	32.42	38.39	6.25	6.25	
Substance 20			45.9	10*	10*	
Substance 21			2.67	10*	1*	
Substance 22	0.056	0.33		0.1	0.1	
Substance 23	1.57	2.11	3.73	3.13*	0.78*	
Substance 24	0.912	1.083	1.505	1.1	1.1	
Substance 25						
Substance 26		74.6	6.7	1	1	
Substance 27				0.01	0.01	
Substance 28	15.8	67.96		32	32	
Substance 29		47.86				0.36
Substance 30				0	0	
Substance 31	0.036	0.057	0.136	0.025	0.025	
Substance 32				0.32	0.18	
Substance 33	3.824	4.628	6.668	3.125*	3.125*	
Substance 34				0.3	0.3	
Substance 35				57	17	
Substance 36				0.316	0.316	
Substance 37				0.003	0.003	
Substance 38	0.305	0.366	0.521	0.38	0.38	
48 h						
Substance 1	2.8	9.1	88.2	3.2	3.2	
Substance 2	0.369	0.451	0.662	0.21	0.1	
Substance 3	0.361	0.45	0.687	0.21	0.1	
Substance 4		0.013	2.757			
Substance 5	1.03	13.18		25	6.25	
Substance 6	1.097	2.694	15.035	0.156	0.156	
Substance 7	4.47	10.25	50.13			31.6
Substance 8	0.005	0.02	0.276	0.0075	0.0075	
Substance 9				8*	8*	
Substance 10	0.478	0.6419	1.1287	0.125	0.125	

Table A8 (continued)

	EC_{10}	EC_{20}	EC_{50}	NOEC D	NOEC W	NOEC B
Substance 11			34.4	32*	32*	
Substance 12	0.0005	0.0015	0.0114	0.001	0.001	
Substance 13	0.09	0.4	7.23	0.1	0.1	
Substance 14	0.12	0.87		1.1	0.5	
Substance 15	0.073	0.757	66.282	0.5	0.158	
Substance 16				0	0.1	
Substance 17	0.022	0.043	0.155	0.032	0.032	
Substance 18	0.005	0.022	0.316	0.0125*	0.0125*	
Substance 19			39.35	25	25	
Substance 20			43.8	10*	10*	
Substance 21	4.06	8.66	36.93	1*	1*	
Substance 22	0.062	0.203	1.999	0.1*	0.05*	
Substance 23	2.64	3.05	4.03	1.56	1.56	
Substance 24	0.034	0.17		0.37	0.37	
Substance 25		0.1	0.5			
Substance 26	0.3	1	12.2			
Substance 27				0.01	0.01	
Substance 28	16.18	68.57		10	3.2	
Substance 29	0.66	6.42	500.54	0.36	0.36	
Substance 30	0.436	3.38		0.716	0.716	
Substance 31	0.042	0.066	0.158	0.05	0.025	
Substance 32	4.06			0.32	0.32	
Substance 33	4.78	8.616	26.608	3.125	3.125	
Substance 34			19.36	0.3	0.3	
Substance 35				1.7	1.7	
Substance 36	0.02	0.192				0.01
Substance 37	0.0007	0.0119		0.003	0.003	
Substance 38	0.049	0.225	4.21	0.38	0.12	
72 h						
Substance 1	0.3	5.2		1	1	
Substance 2	0.361	0.432	0.608	0.21*	0.21*	
Substance 3	0.351	0.427	0.624	0.21*	0.21*	
Substance 4				1	1	
Substance 5	1.72	6.48	82.39	1.6*	1.6*	
Substance 6	1.166	2.968	17.751	0.625	0.625	
Substance 7	33.05	36.13	42.85	31.6	31.6	
Substance 8	0.068	0.086	0.135	0.015*	0.015*	
Substance 9	11.6	2.7		8*	8*	
Substance 10	0.3905	0.5365	0.9853	0.25	0.125	
Substance 11	147.8			32	32	
Substance 12	0.0013	0.0035	0.0225	0.001	0.001	
Substance 13	0.34	1.68		0.22	0.22	
Substance 14	0.08	1.91		0.5	0.5	
Substance 15	0.066	1.144	267.375	0.158	0.158	
Substance 16	13.934	20.965	45.814	10*	10*	
Substance 17	0.061	0.095	0.217	0.056	0.056	
Substance 18	0.019	0.228		0.0125	0.0125	
Substance 19			42.01	25	25	
Substance 20			26.37	10*	10*	
Substance 21			34.94	10*	10*	
Substance 22	0.094	0.244	1.535	0.1*	0.1*	
Substance 23	2.54	3.14	4.7	1.56	1.56	
Substance 24	0.818			0.37	0.23	
Substance 25	0.1	0.1	0.2	0.1	0.1	

Table A8 (continued)

	EC_{10}	EC_{20}	EC_{50}	NOEC D	NOEC W	NOEC B
Substance 26	1.1	2.5	12.1			
Substance 27			0.0078	0.01*	0.01*	
Substance 28	10.96	65.57		1	1	
Substance 29	1.21	6.69	176.94	0.36	0.36	
Substance 30	0.327	8.738		0.411	0.411	
Substance 31	0.069	0.102	0.213	0.05	0.05	
Substance 32				0	0	
Substance 33	12.419	16.773	29.812	3.125	3.125	
Substance 34			14.43	0.3	0.3	
Substance 35				0.47	0.47	
Substance 36	0.01	0.06				0.032
Substance 37	0.0026	0.0173	0.6329	0.003	0.003	
Substance 38	0.406	1.423	15.664	0.38	0.12	
96 h						
Substance 1	0.3	3.8		1	1	
Substance 4				1	0.5	
Substance 5	2.11	10.56	230.42	1.6*	1.6*	
Substance 8	0.051	0.067	0.111	0.028*	0.028*	
Substance 10	0.4039	0.5516	1.0017	0.25	0.125	
Substance 12	0.003	0.0065	0.0286	0.0032	0.0032	
Substance 16	2.736	6.823	39.217	10	10	
Substance 19			41.65	25	25	
Substance 22	0.145	0.348	1.86	0.1*	0.1*	
Substance 23	1.73	2.84	7.29	1.56	0.78	
Substance 24	0.363	1.273		0.13	0.054	
Substance 27			0.0128	0.01	0.01	
Substance 33	12.498	21.723	62.567	6.25	6.25	
Substance 34		1.05		0.3	0.3	
Substance 36	0.007	0.04	1.17			0.032
Substance 37	0.0013	0.0068	0.1732	0.003	0.003	

Table A9: Toxicity parameters for “Growth Rate*”; NOEC D: NOEC from Dunnett’s test; NOEC W: NOEC from Williams Test; NOEC B: NOEC from Bonferroni-U test; values marked with an asterisk indicate lacking prerequisites for the statistical test, rest see text

	EC_{10}	EC_{20}	EC_{50}	NOEC D	NOEC W	NOEC B
24 h						
Substance 1	0.5	10.4		32	1	
Substance 2	0.039	0.099	0.591	0.046	0.046	
Substance 3	0.247	0.366	0.776	0.21	0.21	
Substance 4				1*	1*	
Substance 5				1.6	1.6	
Substance 6	0.041	0.218	5.38			
Substance 7			97.57	56.2	56.2	
Substance 8	0.013	0.026	0.095	0.057	0.057	
Substance 9		0.1		8*	8*	
Substance 10	0.1975	0.3089	0.7271	0.0625	0.0625	
Substance 11			31.6	32*	32*	
Substance 12		0.0001	0.0036	0.001	0.001	

Table A9 (continued)

	EC_{10}	EC_{20}	EC_{50}	NOEC D	NOEC W	NOEC B
Substance 13	0.23	0.82	9.4	0.46	0.1	
Substance 14	0.31	5.58		1.1	1.1	
Substance 15		0.229	56.058	0.5	0.158	
Substance 16	0.853	0.312		0.1	0.1	
Substance 17	0.021	0.046	0.205	0.056	0.032	
Substance 18	0.005	0.014	0.087	0.025*	0.025*	
Substance 19	29.68	32.42	38.39	6.25	6.25	
Substance 20			45.9	10*	10*	
Substance 21			2.67	10*	1*	
Substance 22	0.056	0.33		0.1	0.1	
Substance 23	1.57	2.11	3.73	3.13*	0.78*	
Substance 24	0.912	1.083	1.505	1.1	1.1	
Substance 25						
Substance 26		74.6	6.7	1	1	
Substance 27				0.01	0.01	
Substance 28	15.8	67.96		32	32	
Substance 29		47.86				0.36
Substance 30						
Substance 31	0.036	0.057	0.136	0.025	0.025	
Substance 32				0.32	0.18	
Substance 33	3.824	4.628	6.668	3.125*	3.125*	
Substance 34				0.3	0.3	
Substance 35				57	17	
Substance 36				0.316*	0.316*	
Substance 37				0.003	0.003	
Substance 38	0.305	0.366	0.521	0.38	0.38	
48 h						
Substance 1	4.2	8.7	33.9	10	10	
Substance 2			0.676	0.46*	0.46*	
Substance 3	0.427	0.488	0.63	0.21*	0.1*	
Substance 4						
Substance 5	299.92	100.63	12.45			
Substance 6			31.988			
Substance 7		1.59	7.84	10	10	
Substance 8		0.174	0.026	0.015	0.015	
Substance 9						
Substance 10				0.5	0.5	
Substance 11			38	32*	32*	
Substance 12	0.0042	0.0077	0.0253	0.01	0.01	
Substance 13	0.14	2.52		0.22	0.22	
Substance 14		0.35		0.5	0.5	
Substance 15	0.169	1.058	35.289	1.58	1.58	
Substance 16			8.051	3.162*	3.162*	
Substance 17			0.369	0.032	0.032	
Substance 18	0.051	0.725		0.2*	0.1*	
Substance 19			53.95	25	25	
Substance 20	18.02	33.15	106.48	10*	10*	
Substance 21	14.02	24.29	69.47	10*	10*	
Substance 22	0.015	0.028	0.089	0.1	0.1	
Substance 23	1.17	3.15	21.18	3.13	3.13	
Substance 24	0.151	0.256	0.702	0.37	0.37	
Substance 25				0.2*	0.2*	
Substance 26	1.1	2.5	11.2			
Substance 27		0.0041		0.01*	0.01*	

Table A9 (continued)

	EC_{10}	EC_{20}	EC_{50}	NOEC D	NOEC W	NOEC B
Substance 28	3.44	38.35		0.32	0.32	
Substance 29	4.24	12.47	98.16	0.36	0.36	
Substance 30	0.037	1.073		1.018	0.411	
Substance 31	0.029	0.054	0.173	0.05	0.05	
Substance 32	3.33			1.8	1	
Substance 33				25	25	
Substance 34						
Substance 35						
Substance 36	0.01	0.014	0.027	0.01	0.01	
Substance 37		0.0005	0.0085	0.003	0.003	
Substance 38	0.004	0.264		1.2	1.2	
72 h						
Substance 1		25.2		1*	1*	
Substance 2		0.23	0.513	0.1*	0.1*	
Substance 3	0.427	0.378	0.611	0.21*	0.21*	
Substance 4						
Substance 5	299.92	2.64	46.16	6.25	1.6	
Substance 6		1.109	13.91			
Substance 7				31.6	31.6	
Substance 8			0.111	0.057*	0.057*	
Substance 9				8*	8*	
Substance 10		0.3705	0.6836	0.125	0.125	
Substance 11				32*	32*	
Substance 12	0.0042	0.0016	0.02	0.001	0.001	
Substance 13	0.14	5.79	22.73	4.6	2.2	
Substance 14				0.5	0.5	
Substance 15	0.169	1.566		0.158	0.158	
Substance 16		0.56		0.1	0.1	
Substance 17		0.088	0.241	0.1	0.1	
Substance 18	0.051					
Substance 19			40.94	25	25	
Substance 20	18.02		20.94	10*	10*	
Substance 21	14.02		46.29	10*	10*	
Substance 22	0.015	0.278	2.171	0.1*	0.1*	
Substance 23	1.17	2.26	5.26	0.78*	0.78*	
Substance 24	0.151	0.493				
Substance 25						
Substance 26	1.1	0.1	10.3			
Substance 27			0.0108	0.01*	0.01*	
Substance 28	3.44	74.27		10	10	
Substance 29	4.24	6.45	409.02	0.36*	0.36*	
Substance 30	0.037					
Substance 31	0.029	0.121	0.226	0.05	0.025	
Substance 32	3.33					
Substance 33		7.352	15.524	3.125	3.125	
Substance 34			2.19	0.3	0.3	
Substance 35				57	57	
Substance 36	0.01	0.353		0.032	0.01	
Substance 37		1.3471		0.1	0.1	
Substance 38	0.004	0.511	12.103	0.38	0.38	
96 h						
Substance 1	2.9	6.7	33.1	10	10	
Substance 4						

Table A9 (continued)

	EC_{10}	EC_{20}	EC_{50}	NOEC D	NOEC W	NOEC B
Substance 5				6.25*	6.25*	
Substance 8			0.069	0.015*	0.015*	
Substance 10	1.2794			0.5	0.5	
Substance 12	0.0035	0.0078	0.0366	0.01	0.01	
Substance 16			12.085	10	10	
Substance 19	30.32	34.2	43.06	25	25	
Substance 22	0.23	0.337	0.704	0.2	0.1	
Substance 23	3.14			3.13*	3.13*	
Substance 24	0.01	0.056	1.414	0.37	0.37	
Substance 27	0.0027	0.0083	0.0703	0.01	0.01	
Substance 33				3.125	3.125	
Substance 34				0.3	0.3	
Substance 36	0.004	0.009	0.035	0.01	0.01	
Substance 37	0.0007	0.0015	0.0067	0.003	0.003	

A3 Toxicity parameter values obtained with the scenarios of dissimilar distribution of the data points

Table A10: Effect of the selection and spacing of test concentrations in the moderate-slope dos/response scenario on the EC_{50} of the response variables and on the EC_{50}-ratio between the growth rate and the biomass parameter; concentrations were chosen such that they caused either inhibition below or above the true EC_{50} or that they bracketed the EC_{50}; various variability was assumed for the precision of the cell counts (CV = 0%, 5%, 10% and 20%; CV: coefficient of variation in cell count)

	Selected concentrations cause inhibitions				
CV 0%	<< EC_{50}	<= EC_{50}	< EC_{50} >	>= EC_{50}	>> EC_{50}
E_cC_{50}	0.803	0.793	0.799	0.214	0.121
E_bC_{50}	0.948	0.937	0.946	0.465	0.289
E_rC_{50}	9.994	10.005	9.997	10	7.816
$E_{r*}C_{50}$	9.994	10.005	9.997	10	3.923
E_rC_{50}/E_cC_{50}	12.4	12.6	12.5	46.7	64.6
E_rC_{50}/E_bC_{50}	10.5	10.7	10.6	21.5	27.0

	Selected concentrations cause inhibitions				
CV 5%	<< EC_{50}	<= EC_{50}	< EC_{50} >	>= EC_{50}	>> EC_{50}
E_cC_{50}	0.944	0.632	0.8	0.106	0.031
E_bC_{50}	1.086	0.831	0.947	0.274	0.141
E_rC_{50}	12.121	10.273	9.347	8.449	8.089
$Er*C_{50}$	12.915	9.751	9.27	8.251	5.683
E_rC_{50}/E_cC_{50}	12.8	16.3	11.7	79.7	260.9
E_rC_{50}/E_bC_{50}	11.2	12.4	9.9	30.8	57.4

Table A10 (continued)

CV 10%	Selected concentrations cause inhibitions				
	<< EC_{50}	<= EC_{50}	< EC_{50} >	>= EC_{50}	>> EC_{50}
E_cC_{50}	1.139	0.756	0.736	0.121	0.05
E_bC_{50}	1.255	0.975	0.984	0.289	0.205
E_rC_{50}	13.379	12.279	11.322	7.816	9.697
$E_{r*}C_{50}$	26.172	10.369	9.592	3.923	10.155
E_rC_{50}/E_cC_{50}	11.7	16.2	15.4	64.6	193.9
E_rC_{50}/E_bC_{50}	10.7	12.6	11.5	27.0	47.3

CV 20%	Selected concentrations cause inhibitions				
	<< EC_{50}	<= EC_{50}	< EC_{50} >	>= EC_{50}	>> EC_{50}
E_cC_{50}	0.374	0.506	1.102	15.867	0.036
E_bC_{50}	0.349	0.75	1.299	25.166	0.027
E_rC_{50}	47.156	29.282	9.621	464.019	5.684
Er^*C_{50}	13.455	271674.969	4.711	139.395	0.033
E_rC_{50}/E_cC_{50}	126.1	57.9	8.7	29.2	157.9
E_rC_{50}/E_bC_{50}	135.1	39.0	7.4	18.4	210.5

Table A11: Effect of the selection and spacing of test concentrations in the flat-slope dos/response scenario on the EC_{50} of the response variables and on the EC_{50}-ratio between the growth rate and the biomass parameter; concentrations were chosen such that they caused either inhibition below or above the true EC_{50} or that they bracketed the EC_{50}; various variability was assumed for the precision of the cell counts (CV = 0%, 5%, 10% and 20%; CV: coefficient of variation in cell count)

CV 0%	Selected concentrations cause inhibitions				
	<< EC_{50}	<= EC_{50}	< EC_{50} >	>= EC_{50}	>> EC_{50}
E_cC_{50}	0.292	0.288	0.292	0.046	0.006
E_bC_{50}	0.368	0.363	0.369	0.136	0.084
E_rC_{50}	9.998	10.004	9.991	10	10
$E_{r*}C_{50}$	9.998	10.004	9.991	10	10
E_rC_{50}/E_cC_{50}	34.2	34.7	34.2	217.4	1666.7
E_rC_{50}/E_bC_{50}	27.2	27.6	27.1	73.5	119.0

CV 5%	Selected concentrations cause inhibitions				
	<< EC_{50}	<= EC_{50}	< EC_{50} >	>= EC_{50}	>> EC_{50}
E_cC_{50}	0.368	0.278	0.276	0.031	0.006
E_bC_{50}	0.445	0.374	0.379	0.098	0.06
E_rC_{50}	11.996	11.411	10.968	8.592	9.841
Er^*C_{50}	16.242	10.368	9.633	6.398	10.129
E_rC_{50}/E_cC_{50}	32.6	41.0	39.7	277.2	1640.2

Table A11 (continued)

E_rC_{50}/E_bC_{50}	27.0	30.5	28.9	87.7	164.0

	Selected concentrations cause inhibitions				
CV 10%	<< EC_{50}	<= EC_{50}	< EC_{50} >	>= EC_{50}	>> EC_{50}
E_cC_{50}	0.477	0.269	0.26	0.021	0.006
E_bC_{50}	0.545	0.384	0.39	0.07	0.043
E_rC_{50}	15.052	13.325	11.893	7.083	9.591
$E_{r*}C_{50}$	38.474	10.508	9.425	2.698	10.237
E_rC_{50}/E_cC_{50}	31.6	49.5	45.7	337.3	1598.5
E_rC_{50}/E_bC_{50}	27.6	34.7	30.5	101.2	223.0

	Selected concentrations cause inhibitions				
CV 20%	<< EC_{50}	<= EC_{50}	< EC_{50} >	>= EC_{50}	>> EC_{50}
E_cC_{50}	0.473	0.253	0.23	0.008	0.008
E_bC_{50}	0.843	0.406	0.412	0.034	0.023
E_rC_{50}	30.21	20.269	13.539	3.94	8.683
Er*C_{50}	n.d.	9.515	8.568	n.d.	9.737
E_rC_{50}/E_cC_{50}	63.9	80.1	58.9	492.5	1085.4
E_rC_{50}/E_bC_{50}	35.8	49.9	32.9	115.9	377.5

Table A12: Effect of the selection and spacing of test concentrations in the steep-slope dos/response scenario on the EC_{50} of the response variables and on the EC_{50}-ratio between the growth rate and the biomass parameter; concentrations were chosen such that they caused either inhibition below or above the true EC_{50} or that they bracketed the EC_{50}; various variability was assumed for the precision of the cell counts (CV = 0%, 5%, 10% and 20%; CV: coefficient of variation in cell count)

	Selected concentrations cause inhibitions				
CV 0%	<< EC_{50}	<= EC_{50}	< EC_{50} >	>= EC_{50}	>> EC_{50}
E_cC_{50}	3.657	3.633	3.675	2.15	1.176
E_bC_{50}	3.908	3.883	3.929	2.929	2.55
E_rC_{50}	9.98	10.001	10.018	10	10.001
$E_{r*}C_{50}$	n.d.	10.001	10.018	n.d.	10.001
E_rC_{50}/E_cC_{50}	2.7	2.8	2.7	4.7	8.5
E_rC_{50}/E_bC_{50}	2.6	2.6	2.5	3.4	3.9

	Selected concentrations cause inhibitions				
CV 5%	<< EC_{50}	<= EC_{50}	< EC_{50} >	>= EC_{50}	>> EC_{50}
E_cC_{50}	3.731	3.565	3.925	2.248	1.271
E_bC_{50}	3.925	3.786	4.456	2.926	2.045
E_rC_{50}	9.987	10.342	31.458	9.444	9.612

Table A12 (continued)

Er^*C_{50}	n.d.	10.647	30.273	n.d.	9.474
E_rC_{50}/E_cC_{50}	2.7	2.9	8.0	4.2	7.6
E_rC_{50}/E_bC_{50}	2.5	2.7	7.1	3.2	4.7

	Selected concentrations cause inhibitions				
CV 10%	<< EC_{50}	<= EC_{50}	< EC_{50} >	>= EC_{50}	>> EC_{50}
E_cC_{50}	3.671	3.456	3.73	1.419	1.858
E_bC_{50}	3.786	3.749	4.297	1.791	2.169
E_rC_{50}	12.011	10.808	34.016	8.584	9.583
$E_{r^*}C_{50}$	n.d.	10.47	31.359	n.d.	9.656
E_rC_{50}/E_cC_{50}	3.3	3.1	9.1	6.0	5.2
E_rC_{50}/E_bC_{50}	3.2	2.9	7.9	4.8	4.4

	Selected concentrations cause inhibitions				
CV 20%	<< EC_{50}	<= EC_{50}	< EC_{50} >	>= EC_{50}	>> EC_{50}
E_cC_{50}	3.482	3.35	3.241	3.859	0.672
E_bC_{50}	3.833	3.451	3.872	2.096	1.17
E_rC_{50}	13.525	12.004	43.402	9.006	4.222
Er^*C_{50}	n.d.	13.733	35.767	n.d.	1.746
E_rC_{50}/E_cC_{50}	3.9	3.6	13.4	2.3	6.3
E_rC_{50}/E_bC_{50}	3.5	3.5	11.2	4.3	3.6

A4 Toxicity parameter values obtained with the scenarios identified as basic for the submitted tests

Table A13: Scenario 1: Effects of the growth pattern on the EC_{50} of the response variables and on the EC_{50}-ratio between the growth rates and the biomass parameters; the moderate-slope response-curve was used

	24 h	48 h	72 h	96 h
E_cC_{50}	7.603	1.554	0.799	0.532
E_bC_{50}	3.273	1.692	0.946	0.61
E_rC_{50}	9.997	9.997	9.997	9.997
$E_{r^*}C_{50}$	9.997	9.997	9.997	9.997
E_rC_{50}/E_cC_{50}	1.3	6.4	12.5	18.8
E_rC_{50}/E_bC_{50}	3.1	5.9	10.6	16.4
$E_{r^*}C_{50}/E_cC_{50}$	1.3	6.4	12.5	18.8
$E_{r^*}C_{50}/E_bC_{50}$	3.1	5.9	10.6	16.4

Table A14: Scenario 2: Effects of the growth pattern on the EC_{50} of the response variables and on the EC_{50}-ratio between the growth rates and the biomass parameters; the moderate-slope response-curve was used

	24 h	48 h	72 h	96 h
E_cC_{50}	7.603	2.389	0.799	0.532
E_bC_{50}	3.273	2.332	0.928	0.603
E_rC_{50}	9.997	9.997	9.997	9.997
Er^*C_{50}	9.997	9.997	9.997	9.997
E_rC_{50}/E_cC_{50}	1.3	4.2	12.5	18.8
E_rC_{50}/E_bC_{50}	3.1	4.3	10.8	16.6
$E_{r*}C_{50}/E_cC_{50}$	1.3	4.2	12.5	18.8
$E_{r*}C_{50}/E_bC_{50}$	3.1	4.3	10.8	16.6

Table A15: Scenario 3: Effects of the growth pattern on the EC_{50} of the response variables and on the EC_{50}-ratio between the growth rates and the biomass parameters; the moderate-slope response-curve was used

	24 h	48 h	72 h	96 h
E_cC_{50}	7.559	1.528	0.776	4.557
E_bC_{50}	3.246	1.665	0.922	1.325
E_rC_{50}	9.984	9.984	9.984	23.115
$E_{r*}C_{50}$	9.984	9.984	9.984	85.072
E_rC_{50}/E_cC_{50}	1.3	6.5	12.9	5.1
E_rC_{50}/E_bC_{50}	3.1	6.0	10.8	17.4
$E_{r*}C_{50}/E_cC_{50}$	1.3	6.5	12.9	18.7
$E_{r*}C_{50}/E_bC_{50}$	3.1	6.0	10.8	64.2

Table A16: Scenario 4: Effects of the growth pattern on the EC_{50} of the response variables and on the EC_{50}-ratio between the growth rates and the biomass parameters; the moderate-slope response-curve was used

	24 h	48 h	72 h	96 h
E_cC_{50}	1.222	0.293	0.158	0.103
E_bC_{50}	0.493	0.334	0.194	0.119
E_rC_{50}	1.383	2.738	3.399	3.771
Er^*C_{50}	1.383	5.052	2.738	5.052
E_rC_{50}/E_cC_{50}	1.1	9.3	21.5	36.6
E_rC_{50}/E_bC_{50}	2.8	8.2	17.5	31.7
$E_{r*}C_{50}/E_cC_{50}$	1.1	17.2	17.3	49.0
$E_{r*}C_{50}/E_bC_{50}$	2.8	15.1	14.1	42.5

Table A17: Scenario 5: Effects of the growth pattern on the EC_{50} of the response variables and on the EC_{50}-ratio between the growth rates and the biomass parameters; the moderate-slope response-curve was used

	24 h	48 h	72 h	96 h
E_cC_{50}	58.65	2.553	1.028	0.626
E_bC_{50}	0.94	1.602	1.109	0.719
E_rC_{50}	1.748	5.245	6.79	7.427
$E_{r*}C_{50}$	1.748	9.997	5.245	9.997
E_rC_{50}/E_cC_{50}	0.0	2.1	6.6	11.9
E_rC_{50}/E_bC_{50}	1.9	3.3	6.1	10.3
$E_{r*}C_{50}/E_cC_{50}$	0.0	3.9	5.1	16.0
$E_{r*}C_{50}/E_bC_{50}$	1.9	6.2	4.7	13.9

Table A18: Scenario 6: Effects of the growth pattern on the EC_{50} of the response variables and on the EC_{50}-ratio between the growth rates and the biomass parameters; the moderate-slope response-curve was used

	24 h	48 h	72 h	96 h
E_cC_{50}	7.603	1.554	0.799	0.532
E_bC_{50}	4.912	1.917	0.973	0.614
E_rC_{50}	42.967	21.029	16.418	14.498
$Er*C_{50}$	42.967	9.997	21.026	9.997
E_rC_{50}/E_cC_{50}	5.7	13.5	20.5	27.3
E_rC_{50}/E_bC_{50}	8.7	11.0	16.9	23.6
$E_{r*}C_{50}/E_cC_{50}$	5.7	6.4	26.3	18.8
$E_{r*}C_{50}/E_bC_{50}$	8.7	5.2	21.6	16.3

Table A19: Scenario 7: Effects of the growth pattern on the EC_{50} of the response variables and on the EC_{50}-ratio between the growth rates and the biomass parameters; the moderate-slope response-curve was used

	24 h	48 h	72 h	96 h
E_cC_{50}	7.603	1.554	0.799	0.532
E_bC_{50}	1.617	1.328	0.893	0.604
E_rC_{50}	2.572	5.062	6.384	7.157
$E_{r*}C_{50}$	2.572	9.997	5.062	9.997
E_rC_{50}/E_cC_{50}	0.3	3.3	8.0	13.5
E_rC_{50}/E_bC_{50}	1.6	3.8	7.1	11.8
$E_{r*}C_{50}/E_cC_{50}$	0.3	6.4	6.3	18.8
$E_{r*}C_{50}/E_bC_{50}$	1.6	7.5	5.7	16.6

Table A20: Scenario 8: Effects of the growth pattern on the EC_{50} of the response variables and on the EC_{50}-ratio between the growth rates and the biomass parameters; the moderate-slope response-curve was used

	24 h	48 h	72 h	96 h
E_cC_{50}	n.d.	0.007	0.021	0.031
E_bC_{50}	n.d.	0.007	0.018	0.027
E_rC_{50}	0.007	1.214	2.962	4.274
Er^*C_{50}	0.007	9.984	1.213	9.984
E_rC_{50}/E_cC_{50}	n.d.	173.4	141.0	137.9
E_rC_{50}/E_bC_{50}	n.d.	173.4	164.6	158.3
$E_{r*}C_{50}/E_cC_{50}$	n.d.	1426.3	57.8	322.1
$E_{r*}C_{50}/E_bC_{50}$	n.d.	1426.3	67.4	369.8

Table A21: Scenario 9: Effects of the growth pattern on the EC_{50} of the response variables and on the EC_{50}-ratio between the growth rates and the biomass parameters; the moderate-slope response-curve was used

	24 h	48 h	72 h	96 h
E_cC_{50}	7.603	1.554	1.554	1.554
E_bC_{50}	3.273	1.692	1.534	1.543
E_rC_{50}	9.997	9.997	58.396	257.054
$E_{r*}C_{50}$	9.997	9.997	257.054	257.054
E_rC_{50}/E_cC_{50}	1.3	6.4	37.6	165.4
E_rC_{50}/E_bC_{50}	3.1	5.9	38.1	166.6
$E_{r*}C_{50}/E_cC_{50}$	1.3	6.4	165.4	165.4
$E_{r*}C_{50}/E_bC_{50}$	3.1	5.9	167.6	166.6

Table A22: Scenario 10: Effects of the growth pattern on the EC_{50} of the response variables and on the EC_{50}-ratio between the growth rates and the biomass parameters; the moderate-slope response-curve was used

	24 h	48 h	72 h	96 h
E_cC_{50}	7.603	1.339	0.59	0.351
E_bC_{50}	3.273	$1._{50}3$	0.726	0.41
E_rC_{50}	9.997	7.516	5.301	3.849
Er^*C_{50}	9.997	6.086	4.968	3.249
E_rC_{50}/E_cC_{50}	1.3	5.6	9.0	11.0
E_rC_{50}/E_bC_{50}	3.1	5.0	7.3	9.4
$E_{r*}C_{50}/E_cC_{50}$	1.3	4.5	8.4	9.3
$E_{r*}C_{50}/E_bC_{50}$	3.1	4.0	6.8	7.9

Table A23: Scenario 11: Effects of the growth pattern on the EC_{50} of the response variables and on the EC_{50}-ratio between the growth rates and the biomass parameters; the moderate-slope response-curve was used

	24 h	48 h	72 h	96 h
E_cC_{50}	23.52	8.22	6.351	5.489
E_bC_{50}	12.574	8.537	6.741	5.792
E_rC_{50}	26.096	26.096	26.096	26.096
$E_{r*}C_{50}$	26.096	26.096	26.096	26.096
E_rC_{50}/E_cC_{50}	1.1	3.2	4.1	4.8
E_rC_{50}/E_bC_{50}	2.1	3.1	3.9	4.5
$E_{r*}C_{50}/E_cC_{50}$	1.1	3.2	4.1	4.8
$E_{r*}C_{50}/E_bC_{50}$	2.1	3.1	3.9	4.5

Table A24: Scenario 12: Effects of the growth pattern on the EC_{50} of the response variables and on the EC_{50}-ratio between the growth rates and the biomass parameters; the moderate-slope response-curve was used

	24 h	48 h	72 h	96 h
E_cC_{50}	0.676	4.477	2.147	1.212
E_bC_{50}	1.287	5.59	2.917	1.61
E_rC_{50}	4.767	9.386	8.495	7.863
$Er*C_{50}$	4.767	5.052	9.386	5.052
E_rC_{50}/E_cC_{50}	7.1	2.1	4.0	6.5
E_rC_{50}/E_bC_{50}	3.7	1.7	2.9	4.9
$E_{r*}C_{50}/E_cC_{50}$	7.1	1.1	4.4	4.2
$E_{r*}C_{50}/E_bC_{50}$	3.7	0.9	3.2	3.1