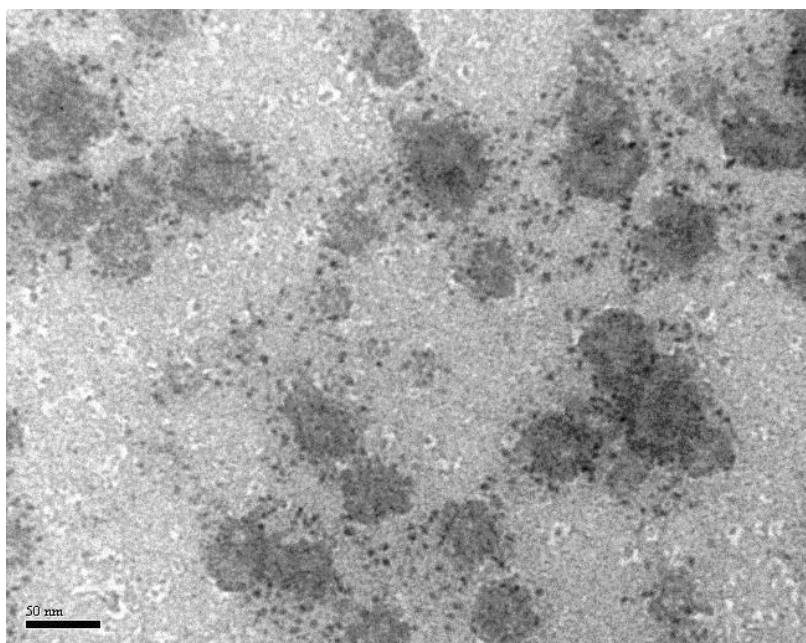
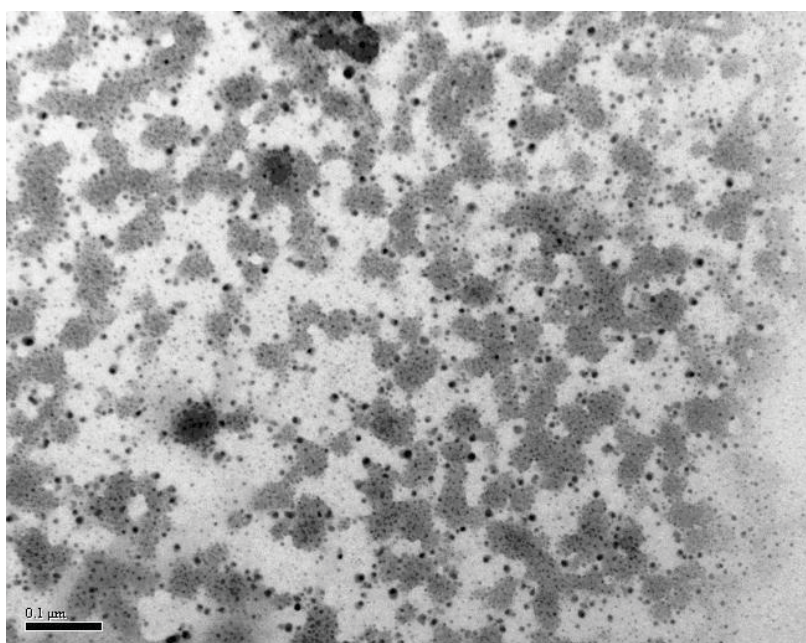




ภาพผนวกที่ ก15 TEM น้ำหลังจากการทดสอบความเป็นพิษของไอออนเงินต่อไรแดง



ภาพผนวกที่ ก16 TEM น้ำหลังจากการทดสอบความเป็นพิษของอนุภาคเงินออกไซด์
ระดับนาโนต่อไรแดง

ภาคผนวก ข**Probit Analysis**

Probit Analysis

วิธีการในทางสถิติที่นิยมใช้ในการหาค่าความเป็นพิษของสารพิษต่อสัตว์ทดลองที่เป็นที่นิยมในปัจจุบัน คือ วิธีโพรบิต (probit analysis) ซึ่งเป็นวิธีการวิเคราะห์ที่พัฒนาโดย Finney (1952) โดยให้สิ่งมีชีวิตหรือสัตว์ทดลองได้รับสารพิษหรือตัวกระตุ้นชนิดใดชนิดหนึ่งแล้วจะพบว่าสิ่งมีชีวิตจะตอบสนองต่อตัวกระตุ้นนั้นโดยที่จำนวนของสิ่งมีชีวิตที่แสดงอาการตอบสนอง (response) ต่อสารพิษจะแตกต่างกันที่ระดับความเข้มข้นต่าง ๆ ซึ่งเรียกการตอบสนองของสัตว์ทดลองนี้ว่า biological variation โดยตัวที่อ่อนแอหรืออ่อนไหวต่อการตอบสนอง (sensitivity) จะแสดงอาการก่อนเมื่อได้รับสารพิษที่ความเข้มข้นระดับต่ำ ๆ ส่วนตัวที่ทนทานต่อสารพิษจะแสดงอาการเมื่อได้รับสารพิษที่ความเข้มข้นระดับสูงขึ้น ความสัมพันธ์ระหว่างความเข้มข้นหรือปริมาณ (dose) และการตอบสนองของสิ่งมีชีวิตจะมีลักษณะเป็นเส้นโค้งในลักษณะซิกมอยด์ (sigmoid curve) เมื่อนำความสัมพันธ์ระหว่างความเข้มข้นของสารพิษกับการตอบสนองของสิ่งมีชีวิต (dose response relationship) มาแจกแจงความถี่จะได้แผนภูมิการแจกแจงเป็นรูปโค้งระฆังคว่ำ (normal frequency distribution) โดยกลุ่มของสิ่งมีชีวิตที่อยู่ทางซ้ายสุดของเส้นโค้งนี้ คือ พวกที่ตอบสนองไวที่สุดและพวกที่อยู่ทางขวาสุดคือพวกที่ทนทานต่อสารพิษ การหาค่า EC_{50} และ LC_{50} จะหาได้จากกราฟเส้นตรง (probit line) ระหว่างความเข้มข้นและค่าโพรบิต โดยเปลี่ยนค่าร้อยละของการตายหรือการตอบสนองเป็นค่าโพรบิตเสียก่อนซึ่งอาจดูจากตารางสำเร็จก็ได้ (ทรรศนีย์, 2539)

อ้างอิงรายละเอียด Probit Analysis อยู่ในเอกสารภาคผนวก ข หน้า 132-141
(<http://userwww.sfsu.edu/efc/classes/biol710/probit/ProbitAnalysis.pdf>)

Probit Analysis

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Quick Overview

- Probit analysis is a type of regression used to analyze binomial response variables.
- It transforms the sigmoid dose-response curve to a straight line that can then be analyzed by regression either through least squares or maximum likelihood.
- Probit analysis can be conducted by one of three techniques:
 - Using tables to estimate the probits and fitting the relationship by eye,
 - Hand calculating the probits, regression coefficient, and confidence intervals, or
 - Having a statistical package such as SPSS do it all for you.

Background

The idea of probit analysis was originally published in *Science* by Chester Ittner Bliss in 1934. He worked as an entomologist for the Connecticut agricultural experiment station and was primarily concerned with finding an effective pesticide to control insects that fed on grape leaves (Greenberg 1980). By plotting the response of the insects to various concentrations of pesticides, he could visually see that each pesticide affected the insects at different concentrations, i.e. one was more effective than the other. However, he didn't have a statistically sound method to compare this difference. The most logical approach would be to fit a regression of the response versus the concentration, or dose and compare between the different pesticides. Yet, the relationship of response to dose was sigmoid in nature and at the time regression was only used on linear data. Therefore, Bliss developed the idea of transforming the sigmoid dose-response curve to a straight line. In 1952, a professor of statistics at the University of Edinburgh by the name of David Finney took Bliss' idea and wrote a book called *Probit Analysis* (Finney 1952). Today, probit analysis is still the preferred statistical method in understanding dose-response relationships.

The Basics

Probit Analysis is a specialized regression model of binomial response variables.

Remember that regression is a method of fitting a line to your data to compare the relationship of the response variable or dependent variable (Y) to the independent variable (X).

$$Y = a + bX + e$$

Where

- a = y-intercept
- b = the slope of the line
- e = error term

Also remember that a binomial response variable refers to a response variable with only two outcomes.

For example:

- Flipping a coin: Heads or tails
- Testing beauty products: Rash/no rash
- The effectiveness or toxicity of pesticides: Death/no death

Applications

Probit analysis is used to analyze many kinds of dose-response or binomial response experiments in a variety of fields. However, because my background knowledge of probit analysis stems only from toxicology, the examples from this webpage will only be of toxicology.

Probit Analysis is commonly used in toxicology to determine the relative toxicity of chemicals to living organisms. This is done by testing the response of an organism under various concentrations of each of the chemicals in question and then comparing the concentrations at which one encounters a response. As discussed above, the response is always binomial (e.g. death/no death) and the relationship between the response and the various concentrations is always sigmoid. Probit analysis acts as a transformation from sigmoid to linear and then runs a regression on the relationship.

Once a regression is run, the researcher can use the output of the probit analysis to compare the amount of chemical required to create the same response in each of the various chemicals. There are many endpoints used to compare the differing toxicities of chemicals, but the LC50 (liquids) or LD50 (solids) are the most widely used outcomes of the modern dose-response experiments. The LC50/LD50 represent the concentration (LC50) or dose (LD50) at which 50% of the population responds.

For example, consider comparing the toxicity of two different pesticides to aphids, pesticide A and pesticide B. If the LC50 of pesticide A is 50ug/L and the LC50 of pesticide B is 10ug/L, pesticide B is more toxic than A because it only takes 10ug/L to kill 50% of the aphids, versus 50ug/L of pesticide A.

How does probit analysis work? How to get from dose-response curve to an LC50?

Below you will find a step by step guide to using probit analysis with various methods. The easiest by far is to use a statistical package such as SPSS, SAS, R, or S, but it is good to see the history of the methodology to get a thorough understanding of the material.

Step 1: Convert % mortality to probits (short for probability unit)

Method A: Determine probits by looking up those corresponding to the % responded in Finney's table (Finney 1952):

Table 3.2 Transformation of percentages to probits

%	0	1	2	3	4	5	6	7	8	9
0	—	2.67	2.95	3.12	3.26	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
—	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

For example, for a 17% response, the corresponding probit would be 4.05. Additionally, for a 50% response (LC50), the corresponding probit would be 5.00.

Method B: Hand calculations (Finney and Stevens 1948):

The probit Y , of the proportion P is defined by:

$$P = \frac{1}{\sqrt{(2\pi)}} \int_{-\infty}^{Y-5} e^{-t^2/2} dt.$$

The standard method of analysis makes use of the maximum and minimum working probits:

$$Y_{\max} = Y + \frac{Q}{Z}$$

$$Y_{\min} = Y - \frac{P}{Z}$$

And the range $1/Z$ where

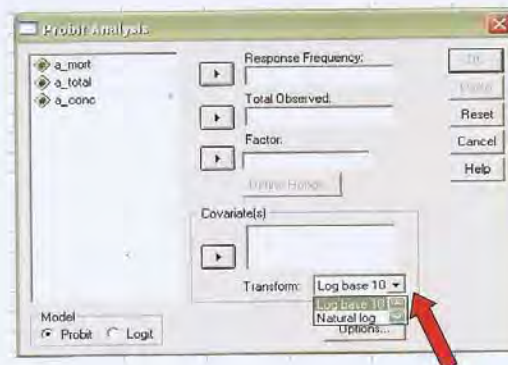
$$Z = \frac{1}{\sqrt{(2\pi)}} e^{-Y^2/2}.$$

Method C: Computer software such as SPSS, SAS, R, or S convert the percent responded to probits automatically.

Step 2: Take the log of the concentrations.

This can either be done by hand if doing hand calculations, or specify this action in the computer program of choice.

For example, after clicking Analyze, Regression, Probit, choose the log of your choice to transform:



Step 3: Graph the probits versus the log of the concentrations and fit a line of regression.

Note: Both least squares and maximum likelihood are acceptable techniques to fitting the regression, but maximum likelihood is preferred because it gives more precise estimation of necessary parameters for correct evaluation of the results (Finney 1952).

Method A: Hand fit the line by eye that minimizes the space between the line and the data (i.e. least squares). Although this method can be surprisingly accurate, calculating a regression by hand or using computer program is obviously more precise. In addition, hand calculations and computer programs can provide confidence intervals.

Method B: Hand calculate the linear regression $Y = \bar{y} + (x - \bar{x})/\sigma$, by using the following method (Finney and Stevens 1948):

First set the proportion responding to be equal to $p = r/n$ and the complement equal to $q = 1 - p$.

The probits of a set value of p should be approximately linearly related to x , the measure of the stimulus, and a line fitted by eye may be used to give a corresponding set of *expected probits*, Y .

The working probit corresponding to each proportion is next calculated from either of the following equations:

$$y = Y + Q/Z - q/Z,$$

$$y = Y - P/Z + p/Z,$$

Next a set of expected probits is then derived from the weighted linear regression equation of working probits on x , each y being assigned a weight, nw , where the weighting coefficient, w , is defined as:

$$w = Z^2/PQ.$$

The process is repeated with the new set of Y values. The iteration converges to give you a linear regression.

Method C: Use a computer program. SPSS uses maximum likelihood to estimate the linear regression.

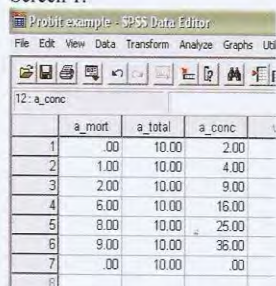
To run the probit analysis in SPSS, follow the following simple steps:

Simply input a minimum of three columns into the Data Editor

- Number of individuals per container that responded
- Total of individuals per container
- Concentrations

For example in the following screen, a_mort is the number of individuals that responded per container, a_total is the total number of individuals per container, and a_conc are the concentrations. Row 7 in the following example is data from the control where 0 out of 10 responded at a concentration of 0.

Screen 1:



	a_mort	a_total	a_conc
1	.00	10.00	2.00
2	1.00	10.00	4.00
3	2.00	10.00	9.00
4	6.00	10.00	16.00
5	8.00	10.00	25.00
6	9.00	10.00	36.00
7	.00	10.00	.00
8			

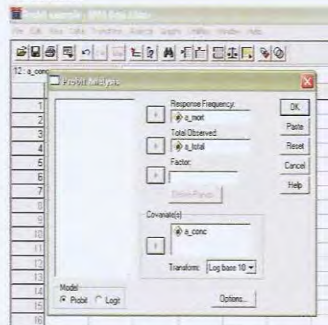
After you columns are set, simply go to analyze, regression, probit:

Screen 2:



Then set your number responded column as the “Response Frequency”, the total number per container as the “Total Observed”, and the concentrations as the “Covariates”. Don’t forget to select the log base 10 to transform your concentrations.

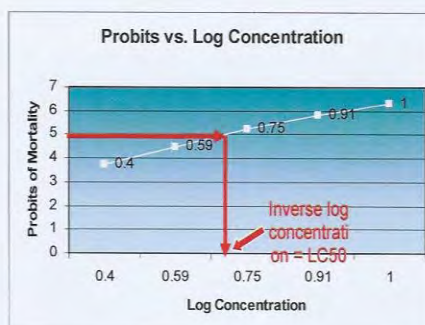
Screen 3:



If you run the above example, you will see that SPSS determines an optimal solution after 18 iterations.

Step 4: Find the LC50

Method A: Using your hand drawn graph, either created by eye or by calculating the regression by hand, find the probit of 5 in the y-axis, then move down to the x-axis and find the log of the concentration associated with it. Then take the inverse of the log and voila! You have the LC50.



Method B: The LC50 is determined by searching the probit list for a probit of 5.00 and then taking the inverse log of the concentration it is associated with.

Step 5: Determine the 95% confidence intervals:

Method A: Hand calculate using the following equation:

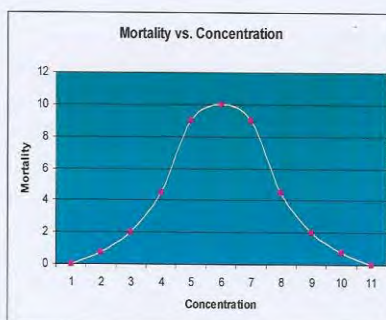
The standard error is approximately: $\pm 1/b \sqrt{(\text{Snw})}$

- b = estimate of the slope of the line
- Snw = summation of nw
- w = weighted coefficient from table III = Z^2/PQ Finney, 1952

Method B: SPSS and other computer programs calculate this automatically

Notes of Interest for Probit Analysis

- Probit analysis assumes that the relationship between number responding (not percent response) and concentration is normally distributed.



If data are not normally distributed, logit (more on this below) is preferred.

- Must correct data if there is more than 10% mortality in the control

One method is to use the Schneider-Orelli's (1947) formula:

$$\text{Corrected} = \frac{\% \text{ Responded} - \% \text{ Responded in Control}}{100 - \% \text{ Responded in Control}} \times 100$$

For example:

Let's say you have 20% mortality in the control and you are correcting the mortality rate for the concentration where 60% occurred. Plug in the mortality rates into the equation above and you come up with a mortality of 50% instead of the original 60%.

$$\frac{60\% - 20\%}{100\% - 20\%} = \frac{40}{80} = 50\%$$

Logit vs. Probit:

Logit is another form of transforming binomial data into linearity and is very similar to probit. Logit functions by taking the log of the odds: $\text{logit}(P) = \log P / (1-P)$. Yet, the relationship between logit and probit is almost indistinguishable: $\text{Logit} \approx (\pi/\sqrt{3}) \times \text{probit}$. In general, if response vs. dose data are not normally distributed, Finney suggests using the logit over the probit transformation (Finney, 1952). Although the multivariate usage of probit analysis is beyond the content of this webpage, it is worth noting that the similarity between probit and logit doesn't hold in a multivariate realm (Hahn and Soyer date unknown). Hahn and Soyer suggest that logit provides a better fit in the presence of extreme independent variable levels and conversely that probit better fit random effects models with moderate data sets (Hahn and Soyer date unknown).

Summary

- Probit Analysis is a type of regression used with binomial response variables. It is very similar to logit, but is preferred when data are normally distributed.
- Most common outcome of a dose-response experiment in which probit analysis is used is the LC50/LD50.
- Probit analysis can be done by eye, through hand calculations, or by using a statistical program.

ภาคผนวก ค

มาตรฐาน OECD สำหรับทดสอบความเป็นพิษต่อสิ่งมีชีวิต

OECD GUIDELINES FOR THE TESTING OF CHEMICALS

Freshwater Alga and Cyanobacteria, Growth Inhibition Test

INTRODUCTION

1. OECD Guidelines for Testing of Chemicals are periodically reviewed and updated in the light of scientific progress. With respect to Guideline 201, Alga, Growth Inhibition Test (adopted June 1984), the need to extend the Guideline to include additional species and update it to meet the requirements for hazard assessment and classification of chemicals has been identified. The revision has been completed on the basis of extensive practical experience, scientific progress in the field of algal toxicity studies, and extensive regulatory use, which has occurred since the original adoption.

2. Definitions used are given in Annex 1.

PRINCIPLE OF THE TEST

3. The purpose of this test is to determine the effects of a substance on the growth of freshwater microalgae and/or cyanobacteria. Exponentially growing test organisms are exposed to the test substance in batch cultures over a period of normally 72 hours. In spite of the relatively brief test duration, effects over several generations can be assessed.

4. The system response is the reduction of growth in a series of algal cultures (test units) exposed to various concentrations of a test substance. The response is evaluated as a function of the exposure concentration in comparison with the average growth of replicate, unexposed control cultures. For full expression of the system response to toxic effects (optimal sensitivity), the cultures are allowed unrestricted exponential growth under nutrient sufficient conditions and continuous light for a sufficient period of time to measure reduction of the specific growth rate.

5. Growth and growth inhibition are quantified from measurements of the algal biomass as a function of time. Algal biomass is defined as the dry weight per volume, e.g. mg algae/litre test solution. However, dry weight is difficult to measure and therefore surrogate parameters are used. Of these surrogates, cell counts are most often used. Other surrogate parameters include cell volume, fluorescence, optical density, etc. A conversion factor between the measured surrogate parameter and biomass should be known.

6. The test endpoint is inhibition of growth, expressed as the logarithmic increase in biomass (average specific growth rate) during the exposure period. From the average specific growth rates recorded in a series of test solutions, the concentration bringing about a specified x % inhibition of growth rate (e.g. 50%) is determined and expressed as the E_xC_x (e.g. $E_{50}C_{50}$).

7. An additional response variable used in this Guideline is yield, which may be needed to fulfil specific regulatory requirements in some countries. It is defined as the biomass at the end of the exposure period minus the biomass at the start of the exposure period. From the yield recorded in a series of test solutions, the concentration bringing about a specified x % inhibition of yield (e.g., 50 %) is calculated and expressed as the E_yC_x (e.g. $E_{50}C_{50}$).

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8. In addition, the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) may be statistically determined.

INFORMATION ON THE TEST SUBSTANCE

9. Information on the test substance which may be useful in establishing the test conditions includes structural formula, purity, stability in light, stability under the conditions of the test, light absorption properties, pKa, and results of studies of transformation including biodegradability in water.

10. The water solubility, octanol water partition coefficient (P_{ow}) and vapour pressure of the test substance should be known and a validated method for the quantification of the substance in the test solutions with reported recovery efficiency and limit of detection should be available.

VALIDITY OF THE TEST

11. For the test to be valid, the following performance criteria should be met:

- The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72-hour test period. This corresponds to a specific growth rate of 0.92 day^{-1} . For the most frequently used species the growth rate is usually substantially higher (see Annex 2). This criterion may not be met when species that grow slower than those listed in Annex 2 are used. In this case, the test period should be extended to obtain at least a 16-fold growth in control cultures, while the growth has to be exponential throughout the test period. The test period may be shortened to at least 48 hours to maintain unlimited, exponential growth during the test as long as the minimum multiplication factor of 16 is reached.
- The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2 and 2-3, for 72-hour tests) in the control cultures (See Annex 1 under "coefficient of variation") must not exceed 35%. See paragraph 49 for the calculation of section-by-section specific growth rate. This criterion applies to the mean value of coefficients of variation calculated for replicate control cultures.
- The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7% in tests with *Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus*. For other less frequently tested species, the value should not exceed 10%.

REFERENCE SUBSTANCE

12. Reference substance(s), such as 3,5-dichlorophenol used in the international ring test (1), may be tested as a means of checking the test procedure. Potassium dichromate can also be used as a reference substance for green algae. It is desirable to test a reference substance at least twice a year.

APPLICABILITY OF THE TEST

13. This Guideline is most easily applied to water-soluble substances which, under the conditions of the test, are likely to remain in the water. For testing of substances that are volatile, strongly adsorbing, coloured, having a low solubility in water or substances that may affect the availability of nutrients or minerals in the test medium, certain modifications of the described procedure may be required (e.g., closed system, conditioning of the test vessels). Guidance on some appropriate modifications is given in (2) (3) and (4).

DESCRIPTION OF THE METHOD

Apparatus

14. Test vessels and other apparatus which will come into contact with the test solutions should be made entirely of glass or other chemically inert material. The items should be thoroughly washed to ensure that no organic or inorganic contaminants may interfere with the algal growth or composition of the test solutions.

15. The test vessels will normally be glass flasks of dimensions that allow a sufficient volume of culture for measurements during the test and a sufficient mass transfer of CO₂ from the atmosphere (see paragraph 30). Note that the liquid volume must be sufficient for analytical determinations (see paragraph 37).

16. In addition some or all of the following equipment may be required:

- Culturing apparatus: a cabinet or chamber is recommended, in which the chosen incubation temperature can be maintained at $\pm 2^{\circ}\text{C}$.
- Light measurement instruments: it is important to note that the method of measurement of light intensity, and in particular the type of receptor (collector), may affect the measured value. Measurements should preferably be made using a spherical (4π) receptor (which responds to direct and reflected light from all angles above and below the plane of measurement), or a 2π receptor (which responds to light from all angles above the measurement plane).
- Apparatus to determine algal biomass. Cell count, which is the most frequently used surrogate parameter for algal biomass, may be made using an electronic particle counter, a microscope with counting chamber, or a flow cytometer. Other biomass surrogates can be measured using a flow cytometer, fluorimeter, spectrophotometer or colorimeter. A conversion factor relating cell count to dry weight is useful to calculate. In order to provide useful measurements at low biomass concentrations when using a spectrophotometer, it may be necessary to use cuvettes with a light path of at least 4 cm.

Test organisms

17. Several species of non-attached microalgae and cyanobacteria may be used. The strains listed in Annex 2 have been shown to be suitable using the test procedure specified in this Guideline.

18. If other species are used, the strain and/or origin should be reported. Confirm that exponential growth of the selected test alga can be maintained throughout the test period under the prevailing conditions.

Growth medium

19. Two alternative growth media, the OECD and the AAP medium, are recommended. The compositions of these media are shown in Annex 3. Note that the initial pH value and the buffering capacity (regulating pH increase) of the two media are different. Therefore the results of the tests may be different depending on the medium used, particularly when testing ionising substances.

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20. Modification of the growth media may be necessary for certain purposes, e.g. when testing metals and chelating agents or testing at different pH values. Use of a modified medium should be described in detail and justified (3)(4).

Initial biomass concentration

21. The initial biomass in the test cultures must be the same in all test cultures and sufficiently low to allow exponential growth throughout the incubation period without risk of nutrient depletion. The initial biomass should not exceed 0.5 mg/L as dry weight. The following initial cell concentrations are recommended:

<i>Pseudokirchneriella subcapitata</i> :	$5 \times 10^3 - 10^4$	cells/mL
<i>Desmodesmus subspicatus</i>	$2-5 \times 10^3$	cells/mL
<i>Navicula pelliculosa</i>	10^4	cells/mL
<i>Anabaena flos-aquae</i>	10^4	cells/mL
<i>Synechococcus leopoliensis</i>	$5 \times 10^4 - 10^5$	cells/mL

Concentrations of test substance

22. The concentration range in which effects are likely to occur may be determined on the basis of results from range-finding tests. For the final definitive test at least five concentrations, arranged in a geometric series with a factor not exceeding 3.2, should be selected. For test substances showing a flat concentration response curve a higher factor may be justified. The concentration series should preferably cover the range causing 5-75 % inhibition of algal growth rate.

Replicates and controls

23. The test design should include three replicates at each test concentration. If determination of the NOEC is not required, the test design may be altered to increase the number of concentrations and reduce the number of replicates per concentration. The number of control replicates must be at least three, and ideally should be twice the number of replicates used for each test concentration.

24. A separate set of test solutions may be prepared for analytical determinations of test substance concentrations (see paragraphs 36 and 38).

25. When a solvent is used to solubilise the test substance, additional controls containing the solvent at the same concentration as used in the test cultures must be included in the test design.

Preparation of inoculum culture

26. In order to adapt the test alga to the test conditions and ensure that the algae are in the exponential growth phase when used to inoculate the test solutions, an inoculum culture in the test medium is prepared 2-4 days before start of the test. The algal biomass should be adjusted in order to allow exponential growth to prevail in the inoculum culture until the test starts. Incubate the inoculum culture under the same conditions as the test cultures. Measure the increase in biomass in the inoculum culture to ensure that growth is within the normal range for the test strain under the culturing conditions. An example of the procedure for algal culturing is described in Annex 4. To avoid synchronous cell divisions during the test a second propagation step of the inoculum culture may be required.

Preparation of test solutions

27. All test solutions must contain the same concentrations of growth medium and initial biomass of test alga. Test solutions of the chosen concentrations are usually prepared by mixing a stock solution of the test substance with growth medium and inoculum culture. Stock solutions are normally prepared by dissolving the substance in test medium.

28. Solvents, e.g. acetone, t-butyl alcohol and dimethyl formamide, may be used as carriers to add substances of low water solubility to the test medium (2)(3). The concentration of solvent should not exceed 100 µl/L, and the same concentration of solvent should be added to all cultures (including controls) in the test series.

Incubation

29. Cap the test vessels with air-permeable stoppers. The vessels are shaken and placed in the culturing apparatus. During the test it is necessary to keep the algae in suspension and to facilitate transfer of CO₂. To this end constant shaking or stirring should be used. The cultures should be maintained at a temperature in the range of 21 to 24°C, controlled at ± 2°C. For species other than those listed in Annex 2, e.g. tropical species, higher temperatures may be appropriate, providing that the validity criteria can be fulfilled. It is recommended to place the flasks randomly and to reposition them daily in the incubator.

30. The pH of the control medium should not increase by more than 1.5 units during the test. For metals and compounds that partly ionise at a pH around the test pH, it may be necessary to limit the pH drift to obtain reproducible and well defined results. A drift of < 0.5 pH units is technically feasible and can be achieved by ensuring an adequate CO₂ mass transfer rate from the surrounding air to the test solution, e.g. by increasing the shaking rate. Another possibility is to reduce the demand for CO₂ by reducing the initial biomass or the test duration.

31. The surface where the cultures are incubated should receive continuous, uniform fluorescent illumination e.g. of «cool-white» or «daylight» type. Strains of algae and cyanobacteria vary in their light requirements. The light intensity should be selected to suit the test organism used. For the recommended species of green algae, select the light intensity at the level of the test solutions from the range of 60-120 µE·m⁻²·s⁻¹ when measured in the photosynthetically effective wavelength range of 400-700 nm using an appropriate receptor. Some species, in particular *Anabaena flos-aquae*, grow well at lower light intensities and may be damaged at high intensities. For such species an average light intensity in the range 40-60 µE·m⁻²·s⁻¹ should be selected. (For light-measuring instruments calibrated in lux, an equivalent range of 4440 – 8880 lux for cool white light corresponds approximately to the recommended light intensity 60-120 µE·m⁻²·s⁻¹). Maintain the light intensity within ±15% from the average light intensity over the incubation area.

Test duration

32. Test duration is normally 72 hours. However, shorter or longer test durations may be used provided that all validity criteria in paragraph 11 can be met.

Measurements and analytical determinations

33. The algal biomass in each flask is determined at least daily during the test period. If measurements are made on small volumes removed from the test solution by pipette, these should not be replaced.

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34. Measurement of biomass is done by manual cell counting by microscope or an electronic particle counter (by cell counts and/or biovolume). Alternative techniques, e.g. flow cytometry, *in vitro* or *in vivo* chlorophyll fluorescence (5) (6), or optical density can be used if a satisfactory correlation with biomass can be demonstrated over the range of biomass occurring in the test.

35. Measure the pH of the solutions at the beginning and at the end of the test.

36. Provided an analytical procedure for determination of the test substance in the concentration range used is available, the test solutions should be analysed to verify the initial concentrations and maintenance of the exposure concentrations during the test.

37. Analysis of the concentration of the test substance at the start and end of the test of a low and high test concentration and a concentration around the expected EC_{50} may be sufficient where it is likely that exposure concentrations will vary less than 20% from nominal values during the test. Analysis of all test concentrations at the beginning and at the end of the test is recommended where concentrations are unlikely to remain within 80-120 % of nominal. For volatile, unstable or strongly adsorbing test substances, additional samplings for analysis at 24 hour intervals during the exposure period are recommended in order to better define loss of the test substance. For these substances, extra replicates may be needed. In all cases, determination of test substance concentrations need only be performed on one replicate vessel at each test concentration (or the contents of the vessels pooled by replicate).

38. The test media prepared specifically for analysis of exposure concentrations during the test should be treated identically to those used for testing, i.e. they should be inoculated with algae and incubated under identical conditions. If analysis of the dissolved test substance concentration is required, it may be necessary to separate algae from the medium. Separation should preferably be made by centrifugation at a low g-force, sufficient to settle the algae.

39. If there is evidence that the concentration of the substance being tested has been satisfactorily maintained within ± 20 % of the nominal or measured initial concentration throughout the test, analysis of the results can be based on nominal or measured initial values. If the deviation from the nominal or measured initial concentration is not within the range of ± 20 %, analysis of the results should be based on geometric mean concentration during exposure or on models describing the decline of the concentration of the test substance (3) (7).

40. The alga growth inhibition test is a more dynamic test system than most other short-term aquatic toxicity tests. As a consequence, the actual exposure concentrations may be difficult to define, especially for adsorbing substances tested at low concentrations. In such cases, disappearance of the test substance from solution by adsorption to the increasing algal biomass does not mean that it is lost from the test system. When the result of the test is analysed, it should be checked whether a decrease in concentration of the test substance in the course of the test is accompanied by a decrease in growth inhibition. If this is the case, application of a suitable model describing the decline of the concentration of the test substance (7) may be considered. If not, it may be appropriate to base the analysis of the results on the initial (nominal or measured) concentrations.

Other observations

41. Microscopic observation should be performed to verify a normal and healthy appearance of the inoculum culture and to observe any abnormal appearance of the algae (as may be caused by the exposure to the test substance) at the end of the test.

Limit test

42. Under some circumstances, e.g. when a preliminary test indicates that the test substance has no toxic effects at concentrations up to 100 mg/L or up to its limit of solubility in the test medium (whichever is the lower), a limit test involving a comparison of responses in a control group and one treatment group (100 mg/L or a concentration equal to the limit of solubility), may be undertaken. It is strongly recommended that this be supported by analysis of the exposure concentration. All previously described test conditions and validity criteria apply to a limit test, with the exception that the number of treatment replicates should be at least six. The response variables in the control and treatment group may be analysed using a statistical test to compare means, e.g. a Student's t-test. If variances of the two groups are unequal, a t-test adjusted for unequal variances should be performed.

DATA AND REPORTING**Plotting growth curves**

43. The biomass in the test vessels may be expressed in units of the surrogate parameter used for measurement (e.g. cell number, fluorescence).

44. Tabulate the estimated biomass concentration in test cultures and controls together with the concentrations of test material and the times of measurement, recorded with a resolution of at least whole hours, to produce plots of growth curves. Both logarithmic scales and linear scales can be useful at this first stage, but logarithmic scales are mandatory and generally give a better presentation of variations in growth pattern during the test period. Note that exponential growth produces a straight line when plotted on a logarithmic scale, and inclination of the line (slope) indicates the specific growth rate.

45. Using the plots, examine whether control cultures grow exponentially at the expected rate throughout the test. Examine all data points and the appearance of the graphs critically and check raw data and procedures for possible errors. Check in particular any data point that seems to deviate by a systematic error. If it is obvious that procedural mistakes can be identified and/or considered highly likely, the specific data point is marked as an outlier and not included in subsequent statistical analysis. (A zero algal concentration in one out of two or three replicate vessels may indicate the vessel was not inoculated correctly, or was improperly cleaned). State reasons for rejection of a data point as an outlier clearly in the test report. Accepted reasons are only (rare) procedural mistakes and not just bad precision. Statistical procedures for outlier identification are of limited use for this type of problem and cannot replace expert judgement. Outliers (marked as such) should preferably be retained among the data points shown in any subsequent graphical or tabular data presentation.

Response variables

46. The purpose of the test is to determine the effects of the test substance on the growth of algae. This Guideline describes two response variables, as member countries have different preferences and regulatory needs. In order for the test results to be acceptable in all member countries, the effects should be evaluated using both response variables (a) and (b) described below.

- (a) Average specific growth rate: this response variable is calculated on the basis of the logarithmic increase of biomass during the test period, expressed per day
- (b) Yield: this response variable is the biomass at the end of the test minus the starting biomass.

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47. It should be noted that toxicity values calculated by using these two response variables are not comparable and this difference must be recognised when using the results of the test. EC_x values based upon average specific growth rate (E_xC_x) will generally be higher than results based upon yield (E_yC_y) if the test conditions of this Guideline are adhered to, due to the mathematical basis of the respective approaches. This should not be interpreted as a difference in sensitivity between the two response variables, simply that the values are different mathematically. The concept of average specific growth rate is based on the general exponential growth pattern of algae in non-limited cultures, where toxicity is estimated on the basis of the effects on the growth rate, without being dependent on the absolute level of the specific growth rate of the control, slope of the concentration-response curve or on test duration. In contrast, results based upon the yield response variable are dependent upon all these other variables. E_yC_y is dependent on the specific growth rate of the algal species used in each test and on the maximum specific growth rate that can vary between species and even different algal strains. This response variable should not be used for comparing the sensitivity to toxicants among algal species or even different strains. While the use of average specific growth rate for estimating toxicity is scientifically preferred, toxicity estimates based on yield are also included in this Guideline to satisfy current regulatory requirements in some countries.

Average growth rate

48. The average specific growth rate for a specific period is calculated as the logarithmic increase in the biomass from the equation for each single vessel of controls and treatments [1]:

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i} \text{ (day}^{-1}\text{)} \text{ ---- [1],}$$

where:

μ_{i-j} is the average specific growth rate from time i to j ;

X_i is the biomass at time i ;

X_j is the biomass at time j

For each treatment group and control group, calculate a mean value for growth rate along with variance estimates.

49. Calculate the average specific growth rate over the entire test duration (normally days 0-3), using the nominally inoculated biomass as the starting value rather than a measured starting value, because in this way greater precision is normally obtained. If the equipment used for biomass measurement allows sufficiently precise determination of the low inoculum biomass (e.g. flow cytometer) then the measured initial biomass concentration can be used. Assess also the section-by-section growth rate, calculated as the specific growth rates for each day during the course of the test (days 0-1, 1-2 and 2-3) and examine whether the control growth rate remains constant (see validity criteria, paragraph 11). A significantly lower specific growth rate on day one than the total average specific growth rate may indicate a lag phase. While a lag phase can be minimised and practically eliminated in control cultures by proper propagation of the pre-culture, a lag phase in exposed cultures may indicate recovery after initial toxic stress or reduced exposure due to loss of test substance (including sorption onto the algal biomass) after initial exposure. Hence the section-by-section growth rate may be assessed in order to evaluate effects of the test substance occurring during the exposure period. Substantial differences between the section-by-section growth rate and the average growth rate indicate deviation from constant exponential growth and that close examination of the growth curves is warranted.

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50. Calculate the percent inhibition of growth rate for each treatment replicate from equation [2]:

$$\%I_r = \frac{\mu_c - \mu_T}{\mu_c} \times 100 \text{ ---- [2],}$$

where:

- %I_r: percent inhibition in average specific growth rate;
- μ_c mean value for average specific growth rate (μ) in the control group;
- μ_T average specific growth rate for the treatment replicate.

51. When solvents are used to prepare the test solutions, the solvent controls rather than the controls without solvents should be used in calculation of percent inhibition.

Yield

52. Yield is calculated as the biomass at the end of the test minus the starting biomass for each single vessel of controls and treatments. For each test concentration and control, calculate a mean value for yield along with variance estimates. The percent inhibition in yield (%I_y) may be calculated for each treatment replicate as follows:

$$\% I_y = \frac{(Y_c - Y_T)}{Y_c} \times 100 \text{ ---- [3]}$$

where:

- % I_y: percent inhibition of yield;
- Y_c: mean value for yield in the control group;
- Y_T: value for yield for the treatment replicate.

Plotting concentration response curve

53. Plot the percentage of inhibition against the logarithm of the test substance concentration and examine the plot closely, disregarding any such data point that was singled out as an outlier in the first phase. Fit a smooth line through the data points by eye or by computerised interpolation to get a first impression of the concentration-response relationship, and then proceed with a more detailed method, preferably a computerised statistical method. Depending on the intended usage of data; the quality (precision) and amount of data as well as the availability of data analysis tools, it may be decided (and sometimes well justified) to stop the data analysis at this stage and simply read the key figures EC₅₀ and EC₁₀ (and/or EC₂₀) from the eye fitted curve (see also section below on stimulatory effects). Valid reasons for not using a statistical method may include:

- Data are not appropriate for computerised methods to produce any more reliable results than can be obtained by expert judgement - in such situations some computer programs may even fail to produce a reliable solution (iterations may not converge etc.)
- Stimulatory growth responses cannot be handled adequately using available computer programs (see below).

Statistical procedures

54. The aim is to obtain a quantitative concentration-response relationship by regression analysis. It is possible to use a weighted linear regression after having performed a linearising transformation of the response data - for instance into probit or logit or Weibull units (8), but non-linear regression procedures are preferred techniques that better handle unavoidable data irregularities and deviations from smooth

distributions. Approaching either zero or total inhibition, such irregularities may be magnified by the transformation, interfering with the analysis (8). It should be noted that standard methods of analysis using probit, logit, or Weibull transforms are intended for use on quantal (e.g. mortality or survival) data, and must be modified to accommodate growth or biomass data. Specific procedures for determination of EC_x values from continuous data can be found in (9) (10) and (11). The use of non-linear regression analysis is further detailed in Annex 5.

55. For each response variable to be analysed, use the concentration-response relationship to calculate point estimates of EC_x values. When possible, the 95% confidence limits for each estimate should be determined. Goodness of fit of the response data to the regression model should be assessed either graphically or statistically. Regression analysis should be performed using individual replicate responses, not treatment group means. If, however nonlinear curve fitting is difficult or fails because of too great scatter in the data, the problem may be circumvented by performing the regression on group means as a practical way of reducing the influence of suspected outliers. Use of this option should be identified in the test report as a deviation from normal procedure because curve fits with individual replicates did not produce a good result.

56. EC_{50} estimates and confidence limits may also be obtained using linear interpolation with bootstrapping (13), if available regression models/methods are unsuitable for the data.

57. For estimation of the LOEC and hence the NOEC, for effects of the test substance on growth rate, it is necessary to compare treatment means using analysis of variance (ANOVA) techniques. The mean for each concentration must then be compared with the control mean using an appropriate multiple comparison or trend test method. Dunnett's or Williams' test may be useful (12)(14)(15)(16)(17). It is necessary to assess whether the ANOVA assumption of homogeneity of variance holds. This assessment may be performed graphically or by a formal test (17). Suitable tests are Levene's or Bartlett's. Failure to meet the assumption of homogeneity of variances can sometimes be corrected by logarithmic transformation of the data. If heterogeneity of variance is extreme and cannot be corrected by transformation, analysis by methods such as step-down Jonkheere trend tests should be considered. Additional guidance on determining the NOEC can be found in (11).

58. Recent scientific developments have led to a recommendation of abandoning the concept of NOEC and replacing it with regression based point estimates EC_x . An appropriate value for x has not been established for this algal test. A range of 10 to 20 % appears to be appropriate (depending on the response variable chosen), and preferably both the EC_{10} and EC_{20} should be reported.

Growth stimulation

59. Growth stimulation (negative inhibition) at low concentrations is sometimes observed. This can result from either hormesis ("toxic stimulation") or from addition of stimulating growth factors with the test material to the minimal medium used. Note that the addition of inorganic nutrients should not have any direct effect because the test medium should maintain a surplus of nutrients throughout the test. Low dose stimulation can usually be ignored in EC_{50} calculations unless it is extreme. However, if it is extreme, or an EC_x value for low x is to be calculated, special procedures may be needed. Deletion of stimulatory responses from the data analysis should be avoided if possible, and if available curve fitting software cannot accept minor stimulation, linear interpolation with bootstrapping can be used. If stimulation is extreme, use of a hormesis model may be considered (18).

Non toxic growth inhibition

60. Light absorbing test materials may give rise to a growth rate reduction because shading reduces the amount of available light. Such physical types of effects should be separated from toxic effects by modifying the test conditions and the former should be reported separately. Guidance may be found in (2) and (3).

Test report

61. The test report must include the following:

Test substance:

- physical nature and relevant physical-chemical properties, including water solubility limit;
- chemical identification data (e.g., CAS Number), including purity (impurities).

Test species:

- the strain, supplier or source and the culture conditions used.

Test conditions:

- date of start of the test and its duration;
- description of test design: test vessels, culture volumes, biomass density at the beginning of the test;
- composition of the medium;
- test concentrations and replicates (e.g., number of replicates, number of test concentrations and geometric progression used);
- description of the preparation of test solutions, including use of solvents etc.
- culturing apparatus;
- light intensity and quality (source, homogeneity);
- temperature;
- concentrations tested: the nominal test concentrations and any results of analyses to determine the concentration of the test substance in the test vessels. The recovery efficiency of the method and the limit of quantification in the test matrix should be reported.;
- all deviations from this Guideline;
- method for determination of biomass and evidence of correlation between the measured parameter and dry weight;

Results:

- pH values at the beginning and at the end of the test at all treatments;
- biomass for each flask at each measuring point and method for measuring biomass;
- growth curves (plot of biomass versus time);
- calculated response variables for each treatment replicate, with mean values and coefficient of variation for replicates;
- graphical presentation of the concentration/effect relationship;
- estimates of toxicity for response variables e.g., EC₅₀, EC₁₀, EC₂₀ and associated confidence intervals. If calculated, LOEC and NOEC and the statistical methods used for their determination;
- if ANOVA has been used, the size of the effect which can be detected (e.g. the least significant difference);
- any stimulation of growth found in any treatment;
- any other observed effects, e.g. morphological changes of the algae;

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- discussion of the results, including any influence on the outcome of the test resulting from deviations from this Guideline.

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- (15) Williams, D.A. (1971). A test for differences between treatment means when several dose levels are compared with a zero dose control. *Biometrics* 27: 103-117.
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ANNEX 1

DEFINITIONS

The following definitions and abbreviations are used for the purposes of this Guideline:

Biomass is the dry weight of living matter present in a population expressed in terms of a given volume; e.g., mg algae/litre test solution. Usually “biomass” is defined as a mass, but in this test this word is used to refer to mass per volume. Also in this test, surrogates for biomass, such as cell counts, fluorescence, etc. are typically measured and the use of the term “biomass” thus refers to these surrogate measures as well.

Coefficient of variation is a dimensionless measure of the variability of a parameter, defined as the ratio of the standard deviation to the mean. This can also be expressed as a percent value. Mean coefficient of variation of average specific growth rate in replicate control cultures should be calculated as follows:

1. Calculate % CV of average specific growth rate out of the daily/section by section growth rates for the respective replicate;
2. Calculate the mean value out of all values calculated under point 1 to get the mean coefficient of variation of the daily/section by section specific growth rate in replicate control cultures.

EC_x is the concentration of the test substance dissolved in test medium that results in an x % (e.g. 50%) reduction in growth of the test organism within a stated exposure period (to be mentioned explicitly if deviating from full or normal test duration). To unambiguously denote an EC value deriving from growth rate or yield the symbol “E_rC” is used for growth rate and “E_yC” is used for yield.

Growth medium is the complete synthetic culture medium in which test algae grow when exposed to the test substance. The test substance will normally be dissolved in the test medium.

Growth rate (average specific growth rate) is the logarithmic increase in biomass during the exposure period.

Lowest Observed Effect Concentration (LOEC) is the lowest tested concentration at which the substance is observed to have a statistically significant reducing effect on growth (at $p < 0.05$) when compared with the control, within a given exposure time. However, all test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC. When these two conditions cannot be satisfied, a full explanation must be given for how the LOEC (and hence the NOEC) has been selected.

No Observed Effect Concentration (NOEC) is the test concentration immediately below the LOEC.

Response variable is a variable for the estimation of toxicity derived from any measured parameters describing biomass by different methods of calculation. For this guideline growth rates and yield are response variables derived from measuring biomass directly or any of the surrogates mentioned.

Specific growth rate is a response variable defined as quotient of the difference of the natural logarithms of a parameter of observation (in this Guideline, biomass) and the respective time period

Yield is the value of a measurement variable at the end of the exposure period minus the measurement variable's value at the start of the exposure period to express biomass increase during the test.

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ANNEX 2

STRAINS SHOWN TO BE SUITABLE FOR THE TEST

Green algae

- *Pseudokirchneriella subcapitata*, (formerly known as *Selenastrum capricornutum*) , ATCC 22662, CCAP 278/4, 61.81 SAG
- *Desmodesmus subspicatus* (formerly known as *Scenedesmus subspicatus*) 86.81 SAG

Diatoms

- *Navicula pelliculosa*, UTEX 664

Cyanobacteria

- *Anabaena flos-aquae*, UTEX 1444, ATCC 29413, CCAP 1403/13A
- *Synechococcus leopoliensis*, UTEX 625, CCAP 1405/1

Sources of Strains

The strains recommended are available in unialgal cultures from the following collections (in alphabetical order):

ATCC: American Type Culture Collection
10801 University Boulevard
Manassas, Virginia 20110-2209
USA

CCAP, Culture Collection of Algae and Protozoa
Institute of Freshwater Ecology,
Windermere Laboratory
Far Sawrey, Ambleside
Cumbria LA22 0LP
UK

SAG: Collection of Algal Cultures
Inst. Plant Physiology
University of Göttingen
Nicholausberger Weg 18
D-3400 Göttingen
GERMANY

UTEX Culture Collection of Algae
Section of Molecular, Cellular and Developmental Biology
School of Biological Sciences
the University of Texas at Austin
Austin, Texas 78712
USA.

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Appearance and characteristics of recommended species

	<i>P. subcapitata</i>	<i>D. subspicatus</i>	<i>N. pelliculosa</i>	<i>A. flos-aquae</i>	<i>S. leopoliensis</i>
Appearance	Curved, twisted single cells	Oval, mostly single cells	Rods	Chains of oval cells	Rods
Size (L x W) μm	8-14 x 2-3	7-15 x 3-12	7.1 x 3.7	4.5 x 3	6 x 1
Cell volume ($\mu\text{m}^3/\text{cell}$)	40-60 ¹	60-80 ¹	40-50 ¹	30-40 ¹	2.5 ²
Cell dry weight (mg/cell)	2-3 x 10 ⁻⁸	3-4 x 10 ⁻⁸	3-4 x 10 ⁻⁸	1-2 x 10 ⁻⁸	2-3 x 10 ⁻⁹
Growth rate ³ (day ⁻¹)	1.5-1.7	1.2-1.5	1.4	1.1-1.4	2.0-2.4

¹ Measured with electronic particle counter

² Calculated from size

³ Most frequently observed growth rate in OECD medium at light intensity approx. 70 $\mu\text{E m}^{-2} \text{s}^{-1}$ and 21 °C

Specific Recommendations on Culturing and Handling of Recommended Test Species*Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus*

These green algae are generally easy to maintain in various culture media. Information on suitable media is available from the culture collections. The cells are normally solitary, and cell density measurements can easily be performed using an electronic particle counter or microscope.

Anabaena flos-aquae

Various growth media may be used for keeping a stock culture. It is particularly important to avoid allowing the batch culture to go past log phase growth when renewing, recovery is difficult at this point.

Anabaena flos-aquae develops aggregates of nested chains of cells. The size of these aggregates may vary with culturing conditions. It may be necessary to break up these aggregates when microscope counting or an electronic particle counter is used for determination of biomass.

Sonication of sub-samples may be used to break up chains to reduce count variability. Longer sonication than required for breaking up chains into shorter lengths may destroy the cells. Sonication intensity and duration must be identical for each treatment.

Count enough fields on the hemocytometer (at least 400 cells) to help compensate for variability. This will improve reliability of microscopic density determinations.

An electronic particle counter can be used for determination of total cell volume of *Anabaena* after breaking up the cell chains by careful sonication. The sonification energy has to be adjusted to avoid disruption of the cells.

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Use a vortex mixer or similar appropriate method to make sure the algae suspension used to inoculate test vessels is well mixed and homogeneous.

Test vessels should be placed on an orbital or reciprocate shaker table at about 150 revolutions per minute. Alternatively, intermittent agitation may be used to reduce the tendency of *Anabaena* to form clumps. If clumping occurs, care must be taken to achieve representative samples for biomass measurements. Vigorous agitation before sampling may be necessary to disintegrate algal clumps.

Synechococcus leopoliensis

Various growth media may be used for keeping a stock culture. Information on suitable media is available from the culture collections.

Synechococcus leopoliensis grows as solitary rod-shaped cells. The cells are very small, which complicates the use of microscope counting for biomass measurements. Electronic particle counters equipped for counting particles down to a size of approximately 1 µm are useful. *In vitro* fluorometric measurements are also applicable.

Navicula pelliculosa

Various growth media may be used for keeping a stock culture. Information on suitable media is available from the culture collections. Note that silicate is required in the medium.

Navicula pelliculosa may form aggregates under certain growth conditions. Due to production of lipids the algal cells sometimes tend to accumulate in the surface film. Under those circumstances special measures have to be taken when sub-samples are taken for biomass determination in order to obtain representative samples. Vigorous shaking, e.g. using a vortex mixer may be required.

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ANNEX 3

GROWTH MEDIA

One of the following two growth media may be used:

OECD medium: Original medium of OECD TG 201, also according to ISO 8692
US. EPA medium AAP also according to ASTM.

When preparing these media, reagent or analytical-grade chemicals should be used and deionised water.

Composition of The AAP-medium (US. EPA) and the OECD TG 201 medium.

Component	AAP		OECD	
	mg/L	mM	mg/L	mM
NaHCO ₃	15.0	0.179	50.0	0.595
NaNO ₃	25.5	0.300		
NH ₄ Cl			15.0	0.280
MgCl ₂ ·6(H ₂ O)	12.16	0.0598	12.0	0.0590
CaCl ₂ ·2(H ₂ O)	4.41	0.0300	18.0	0.122
MgSO ₄ ·7(H ₂ O)	14.6	0.0592	15.0	0.0609
K ₂ HPO ₄	1.044	0.00599		
KH ₂ PO ₄			1.60	0.00919
FeCl ₃ ·6(H ₂ O)	0.160	0.000591	0.0640	0.000237
Na ₂ EDTA·2(H ₂ O)	0.300	0.000806	0.100	0.000269*
H ₃ BO ₃	0.186	0.00300	0.185	0.00299
MnCl ₂ ·4(H ₂ O)	0.415	0.00201	0.415	0.00210
ZnCl ₂	0.00327	0.000024	0.00300	0.0000220
CoCl ₂ ·6(H ₂ O)	0.00143	0.000006	0.00150	0.00000630
Na ₂ MoO ₄ ·2(H ₂ O)	0.00726	0.000030	0.00700	0.0000289
CuCl ₂ ·2(H ₂ O)	0.000012	0.00000007	0.00001	0.00000006
pH	7.5		8.1	

- The molar ratio of EDTA to iron slightly exceed unity. This prevents iron precipitation and at the same time, chelation of heavy metal ions is minimised.

In test with the diatom *Navicula pelliculosa* both media must be supplemented with Na₂SiO₃·9H₂O to obtain a concentration of 1.4 mg Si/L.

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The pH of the medium is obtained at equilibrium between the carbonate system of the medium and the partial pressure of CO₂ in atmospheric air. An approximate relationship between pH at 25 °C and the molar bicarbonate concentration is:

$$\text{pH}_{\text{eq}} = 11.30 + \log[\text{HCO}_3^-]$$

With 15 mg NaHCO₃/L, $\text{pH}_{\text{eq}} = 7.5$ (U.S. EPA medium) and with 50 mg NaHCO₃/L, $\text{pH}_{\text{eq}} = 8.1$ (OECD medium).

Element composition of test media

Element	AAP	OECD
	mg/L	mg/L
C	2.144	7.148
N	4.202	3.927
P	0.186	0.285
K	0.469	0.459
Na	11.044	13.704
Ca	1.202	4.905
Mg	2.909	2.913
Fe	0.033	0.017
Mn	0.115	0.115

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Preparation of OECD medium

Nutrient	Concentration in stock solution
Stock solution 1: macro nutrients	
NH ₄ Cl	1.5 g/L
MgCl ₂ ·6H ₂ O	1.2 g/L
CaCl ₂ ·2H ₂ O	1.8 g/L
MgSO ₄ ·7H ₂ O	1.5 g/L
KH ₂ PO ₄	0.16 g/L
Stock solution 2: iron	
FeCl ₃ ·6H ₂ O	64 mg/L
Na ₂ EDTA·2H ₂ O	100 mg/L
Stock solution 3: trace elements	
H ₃ BO ₃	185 mg/L
MnCl ₂ ·4H ₂ O	415 mg/L
ZnCl ₂	3 mg/L
CoCl ₂ ·6H ₂ O	1.5 mg/L
CuCl ₂ ·2H ₂ O	0.01 mg/L
Na ₂ MoO ₄ ·2H ₂ O	7 mg/L
Stock solution 4: bicarbonate	
NaHCO ₃	50 g/L
Na ₂ SiO ₃ ·9H ₂ O	

Sterilize the stock solutions by membrane filtration (mean pore diameter 0.2 µm) or by autoclaving (120 °C, 15 min). Store the solutions in the dark at 4 °C.

Do not autoclave stock solutions 2 and 4, but sterilise them by membrane filtration.

Prepare a growth medium by adding an appropriate volume of the stock solutions 1-4 to water:

Add to 500 ml of sterilised water:

- 10 ml of stock solution 1
- 1 ml of stock solution 2
- 1 ml of stock solution 3
- 1 ml of stock solution 4

Make up to 1 000 mL with sterilised water.

Allow sufficient time for equilibrating the medium with the atmospheric CO₂, if necessary by bubbling with sterile, filtered air for some hours.

Preparation of U.S. EPA medium

1. Add 1 mL of each stock solution in 2.1–2.7 to approximately 900 mL of deionized or distilled water and then dilute to 1 litre.

2. Macronutrient stock solutions are made by dissolving the following into 500 mL of deionised or distilled water. Reagents 2.1, 2.2, 2.3, and 2.4 can be combined into one stock solution.

- 2.1 NaNO_3 —12.750 g.
- 2.2 $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ —6.082 g.
- 2.3 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ —2.205 g.
- 2.4 *Micronutrient Stock Solution*—(see 3).
- 2.5 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ —7.350 g.
- 2.6 K_2HPO_4 —0.522 g.
- 2.7 NaHCO_3 —7.500 g.
- 2.8 $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ —See Note 1.

NOTE 1: Use for diatom test species only. May be added directly (202.4 mg) or by way of stock solution to give 20 mg/L Si final concentration in medium.

3. The micronutrient stock solution is made by dissolving the following into 500 mL of deionised or distilled water:

- 3.1 H_3BO_3 —92.760 mg.
- 3.2 $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ —207.690 mg.
- 3.3 ZnCl_2 —1.635 mg.
- 3.4 $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ —79.880 mg.
- 3.5 $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ —0.714 mg.
- 3.6 $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ —3.630 mg.
- 3.7 $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ —0.006 mg.
- 3.8 $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ —150.000 mg. [Disodium (Ethylenedinitrilo) tetraacetate].
- 3.9 $\text{Na}_2\text{SeO}_4 \cdot 5\text{H}_2\text{O}$ —0.005 mg See Note 2.

NOTE 2: Use only in medium for stock cultures of diatom species.

4. Adjust pH to 7.5 ± 0.1 with 0.1 N or 1.0 N NaOH or HCl.

5. Filter the media into a sterile container through either a 0.22 μm membrane filter if a particle counter is to be used or a 0.45 μm filter if a particle counter is not to be used.

6. Store medium in the dark at approximately 4°C until use.

ANNEX 4

EXAMPLE OF A PROCEDURE FOR THE CULTURING OF ALGAE

General observations

The purpose of culturing on the basis of the following procedure is to obtain algal cultures for toxicity tests.

Use suitable methods to ensure that the algal cultures are not infected with bacteria. Axenic cultures may be desirable but unialgal cultures must be established and used.

All operations must be carried out under sterile conditions in order to avoid contamination with bacteria and other algae.

Equipment and materials

See under Test Guideline: Apparatus.

Procedures for obtaining algal cultures*Preparation of nutrient solutions (media):*

All nutrient salts of the medium are prepared as concentrated stock solutions and stored dark and cold. These solutions are sterilised by filtration or by autoclaving.

The medium is prepared by adding the correct amount of stock solution to sterile distilled water, taking care that no infection occurs. For solid medium 0.8 per cent of agar is added.

Stock culture:

The stock cultures are small algal cultures that are regularly transferred to fresh medium to act as initial test material. If the cultures are not used regularly they are streaked out on sloped agar tubes. These are transferred to fresh medium at least once every two months.

The stock cultures are grown in conical flasks containing the appropriate medium (volume about 100 ml). When the algae are incubated at 20°C with continuous illumination, a weekly transfer is required.

During transfer an amount of "old" culture is transferred with sterile pipettes into a flask of fresh medium, so that with the fast-growing species the initial concentration is about 100 times smaller than in the old culture.

The growth rate of a species can be determined from the growth curve. If this is known, it is possible to estimate the density at which the culture should be transferred to new medium. This must be done before the culture reaches the death phase.

Pre-culture:

The pre-culture is intended to give an amount of algae suitable for the inoculation of test cultures. The pre-culture is incubated under the conditions of the test and used when still exponentially growing, normally after an incubation period of 2 to 4 days. When the algal cultures contain deformed or abnormal cells, they must be discarded.

ANNEX 5

DATA ANALYSIS BY NONLINEAR REGRESSION

General considerations

The response in algal tests and other microbial growth tests - growth of biomass - is by nature a continuous or metric variable - a process rate if growth rate is used and its integral over time if biomass is selected. Both are referenced to the corresponding mean response of replicate non-exposed controls showing maximum response for the conditions imposed - with light and temperature as primary determining factors in the algal test. The system is distributed or homogenous and the biomass can be viewed as a continuum without consideration of individual cells. The variance distribution of the type of response for a such system relate solely to experimental factors (described typically by the log-normal or normal distributions of error). This is by contrast to typical bioassay responses with quantal data for which the tolerance (typically binomially distributed) of individual organisms are often assumed to be the dominant variance component. Control responses are here zero or background level.

In the uncomplicated situation, the normalized or relative response, r , decreases monotonically from 1 (zero inhibition) to 0 (100 per cent inhibition). Note, that all responses have an error associated and that apparent negative inhibitions can be calculated as a result of random error only.

Regression analysisModels

A regression analysis aims at quantitatively describing the concentration response curve in the form of a mathematical regression function $Y = f(C)$ or more frequently $F(Z)$ where $Z = \log C$. Used inversely $C = f^{-1}(Y)$ allows the calculation of, EC_x figures, including the EC_{50} , EC_{10} and EC_{20} , and their 95% confidence limits. Several simple mathematical functional forms have proved to successfully describe concentration - response relationships obtained in algal growth inhibition tests. Functions include for instance the logistic equation, the nonsymmetrical Weibul equation and the log normal distribution function, which are all sigmoid curves asymptotically approaching one for $C \rightarrow 0$ and zero for $C \rightarrow \text{infinity}$.

The use of continuous threshold function models (e.g. the Kooijman model "for inhibition of population growth" Kooijman et al. 1996) is a recently proposed or alternative to asymptotic models. This model assumes no effects at concentrations below a certain threshold EC_0 that is estimated by extrapolation of the response concentration relationship to intercept the concentration axis using a simple continuous function that is not differentiable in the starting point.

Note that the analysis can be a simple minimization of sums of residual squares (assuming constant variance) or weighted squares if variance heterogeneity is compensated

Procedure

The procedure can be outlined as follows: Select an appropriate functional equation, $Y = f(C)$, and fit it to the data by non-linear regression. Use preferably the measurements from each individual flask rather than means of replicates, in order to extract as much information from the data as possible. If the variance is high, on the other hand, practical experience suggests that means of replicates may provide a more robust mathematical estimation less influenced by systematic errors in the data, than with each individual data point retained.

Plot the fitted curve and the measured data and examine whether the curve fit is appropriate. Analysis of residuals may be a particular helpful tool for this purpose. If the chosen functional relationship to fit the concentration response does not describe well the whole curve or some essential part of it, such as the response at low concentrations, choose another curve fit option - e.g., a non-symmetrical curve like the Weibul function instead of a symmetrical one. Negative inhibitions may be a problem with for instance the

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log - normal distribution function likewise demanding an alternative regression function. It is not recommended to assign a zero or small positive value to such negative values because this distorts the error distribution. It may be appropriate to make separate curve fits on parts of the curve such as the low inhibition part to estimate $EC_{low\ x}$ figures. Calculate from the fitted equation (by "inverse estimation", $C = f^{-1}(Y)$), characteristic point estimates EC_x 's, and report as a minimum the EC_{50} and one or two $EC_{low\ x}$ estimates. Experience from practical testing has shown that the precision of the algal test normally allows a reasonably accurate estimation at the 10 % inhibition level if data points are sufficient - unless stimulation occurs at low concentrations as a confounding factor. The precision of an EC_{20} estimate is often considerably better than that of an EC_{10} , because the EC_{20} is usually positioned on the approximately linear part of the central concentration response curve. Sometimes EC_{10} can be difficult to interpret because of growth stimulation. So while the EC_{10} is normally obtainable with a sufficient accuracy it is recommended to report always also the EC_{20} .

Weighting factors

The experimental variance generally is not constant and typically includes a proportional component, and a weighted regression is therefore advantageously carried out routinely. Weighting factors for a such analysis are normally assumed inversely proportional to the variance:

$$W_i = 1/\text{Var}(r_i)$$

Many regression programs allow the option of weighted regression analysis with weighting factors listed in a table. Conveniently weighting factors should be normalized by multiplying them by $n/\sum w_i$ (n is the number of datapoints) so their sum be one.

Normalizing responses

Normalizing by the mean control response gives some principle problems and gives rise to a rather complicated variance structure. Dividing the responses by the mean control response for obtaining the percentage of inhibition, one introduces an additional error caused by the error on the control mean. Unless this error is negligibly small, weighting factors in the regression and confidence limits must be corrected for the covariance with the control (Draper and Smith, 1981). Note that high precision on the estimated mean control response is important in order to minimize the overall variance for the relative response. This variance is as follows:

(Subscript i refers to concentration level i and subscript 0 to the controls)

$$Y_i = \text{Relative response} = r_i/r_0 = 1 - I = f(C_i)$$

with a variance $\text{Var}(Y_i) = \text{Var}(r_i/r_0) \cong (\partial Y_i / \partial r_i)^2 \cdot \text{Var}(r_i) + ((\partial Y_i / \partial r_0)^2 \cdot \text{Var}(r_0))$

and since $(\partial Y_i / \partial r_i) = 1/r_0$ and $(\partial Y_i / \partial r_0) = r_i/r_0^2$

with normally distributed data and m_i and m_0 replicates: $\text{Var}(r_i) = \sigma^2/m_i$

the total variance of the relative response Y_i thus becomes

$$\text{Var}(Y_i) = \sigma^2/(r_0^2 \cdot m_i) + r_i^2 \cdot \sigma^2/r_0^4 \cdot m_0$$

The error on the control mean is inversely proportional to the square root of the number of control replicates averaged, and sometimes it can be justified to include historic data and in this way greatly reduce the error. An alternative procedure is not to normalize the data and fit the absolute responses including the control response data but introducing the control response value as an additional parameter to be fitted by non linear regression. With a usual 2 parameter regression equation, this method necessitates the fitting of 3 parameters, and therefore demands more data points than non-linear regression on data that are normalized using a pre-set control response .

Inverse confidence intervals

The calculation of non-linear regression confidence intervals by inverse estimation is rather complex and not an available standard option in ordinary statistical computer program packages. Approximate confidence limits may be obtained with standard non-linear regression programs with re-parameterisation (Bruce and Versteeg, 1992), which involves rewriting the mathematical equation with the desired point estimates, e.g. the EC_{10} and the EC_{50} as the parameters to be estimated. (Let the function be $I = f(\alpha, \beta,$

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Concentration) and utilize the definition relationships $f(\alpha, \beta, EC_{10}) = 0.1$ and $f(\alpha, \beta, EC_{50}) = 0.5$ to substitute $f(\alpha, \beta, \text{concentration})$ with an equivalent function $g(EC_{10}, EC_{50}, \text{concentration})$.

A more direct calculation (Andersen et al, 1998) is performed by retaining the original equation and using a Taylor expansion around the means of r_1 and r_0 .

Recently "boot strap methods" have become popular. Such methods use the measured data and a random number generator directed frequent re-sampling to estimate an empirical variance distribution.

Kooijman, S.A.L.M.; Hanstveit, A.O.; Nyholm, N. (1996): No-effect concentrations in algal growth inhibition tests. *Water Research*, 30, 1625-1632.

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13 April 2004**OECD GUIDELINE FOR TESTING OF CHEMICALS*****Daphnia* sp., Acute Immobilisation Test****INTRODUCTION**

1. OECD Guidelines for the Testing of chemicals are periodically reviewed in the light of scientific progress. Guideline 202 on "*Daphnia* sp., Acute Immobilisation Test and Reproduction Test", adopted in April 1984, included two parts: Part I - the 24h EC₅₀ acute immobilisation test and Part II - the reproduction test (at least 14 days). Revision of the reproduction test has resulted in the adoption and publication of Test Guideline 211 on "*Daphnia magna* Reproduction Test" in September 1998. Consequently, the new version of Guideline 202 is restricted to the acute immobilisation test.

2. This guideline describes an acute toxicity test to assess effects of chemicals towards daphnids. Existing test methods were used to the extent possible (1)(2)(3). The main differences in comparison with the earlier version are the extension of the test duration to 48 hours, the provision for more information on recommended culture and test media, and the introduction of a limit test at 100 mg/l of test substance.

PRINCIPLE OF THE TEST

3. Young daphnids, aged less than 24 hours at the start of the test, are exposed to the test substance at a range of concentrations for a period of 48 hours. Immobilisation is recorded at 24 hours and 48 hours and compared with control values. The results are analysed in order to calculate the EC₅₀ at 48h (see Annex 1 for definitions). Determination of the EC₅₀ at 24h is optional.

INFORMATION ON THE TEST SUBSTANCE

4. The water solubility and the vapour pressure of the test substance should be known and a reliable analytical method for the quantification of the substance in the test solutions with reported recovery efficiency, and limit of determination should be available. Useful information includes the structural formula, purity of the substance, stability in water and light, P_{ow} and results of a test for ready biodegradability (see Guideline 301).

Note: Guidance for testing substances with physical chemical properties that make them difficult to test is provided in a separate document (4).

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REFERENCE SUBSTANCES

5. A reference substance may be tested for EC₅₀ as a means of assuring that the test conditions are reliable. Toxicants used in international ring-tests (1)(5) are recommended for this purpose¹. Test(s) with a reference substance should be done preferably every month and at least twice a year.

VALIDITY OF THE TEST

6. For a test to be valid, the following performance criteria apply:
- In the control, including the control containing the solubilising agent, not more than 10 per cent of the daphnids should have been immobilised;
 - The dissolved oxygen concentration at the end of the test should be ≥ 3 mg/l in control and test vessels.

Note: For the first criterion, not more than 10 percent of the control daphnids should show immobilisation or other signs of disease or stress, for example, discoloration or unusual behaviour such as trapping at surface of water.

DESCRIPTION OF THE METHODApparatus

7. Test vessels and other apparatus that will come into contact with the test solutions should be made entirely of glass or other chemically inert material. Test vessels will normally be glass test tubes or beakers; they should be cleaned before each use using standard laboratory procedures. Test vessels should be loosely covered to reduce the loss of water due to evaporation and to avoid the entry of dust into the solutions. Volatile substances should be tested in completely filled closed vessels large enough to prevent oxygen becoming limiting or too low (see paragraphs 6 and 22).

8. In addition some or all of the following equipment will be used: oxygen-meter (with microelectrode or other suitable equipment for measuring dissolved oxygen in low volumes samples); pH-meter; adequate apparatus for temperature control; equipment for the determination of total organic carbon concentration (TOC); equipment for the determination of chemical oxygen demand (COD); equipment for the determination of hardness, etc.

Test organism

9. *Daphnia magna* Straus is the preferred test species although other suitable *Daphnia* species can be used in this test (e.g. *Daphnia pulex*). At the start of the test, the animals should be less than 24 hours old and, to reduce variability, it is strongly recommended they are not first brood progeny. They should be derived from a healthy stock (i.e. showing no signs of stress such as high mortality, presence of males and ephippia, delay in the production of the first brood, discoloured animals, etc.). All organisms used for a

¹ The results of these inter laboratory tests and a Technical Corrigendum to ISO 6341 give an EC₅₀-24 h of the potassium dichromate (K₂Cr₂O₇) within the range 0.6 mg/l to 2.1 mg/l

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particular test should have originated from cultures established from the same stock of daphnids. The stock animals must be maintained in culture conditions (light, temperature, medium) similar to those to be used in the test. If the daphnids culture medium to be used in the test is different from that used for routine daphnids culture, it is good practice to include a pre-test acclimation period. For that, brood daphnids should be maintained in dilution water at the test temperature for at least 48 hours prior to the start of the test.

Holding and dilution water

10. Natural water (surface or ground water), reconstituted water or dechlorinated tap water are acceptable as holding and dilution water if daphnids will survive in it for the duration of the culturing, acclimation and testing without showing signs of stress. Any water which conforms to the chemical characteristics of an acceptable dilution water as listed in Annex 2 is suitable as a test water. It should be of constant quality during the period of the test. Reconstituted water can be made up by adding specific amounts of reagents of recognised analytical grade to deionised or distilled water. Examples of reconstituted water are given in (1)(6) and in Annex 3. Note that media containing known chelating agents, such as M4 and M7 media in Annex 3, should be avoided for testing substances containing metals. The pH should be in the range of 6 to 9. Hardness between 140 and 250 mg/l (as CaCO₃) is recommended for *Daphnia magna*, while lower hardness may be also appropriate for other *Daphnia* species. The dilution water may be aerated prior to use for the test so that the dissolved oxygen concentration has reached saturation.

11. If natural water is used, the quality parameters should be measured at least twice a year or whenever it is suspected that these characteristics may have changed significantly (see paragraph 10 and Annex 2). Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni) should also be made. If dechlorinated tap water is used, daily chlorine analysis is desirable. If the dilution water is from a surface or ground water source, conductivity and total organic carbon (TOC) or chemical oxygen demand (COD) should be measured.

Test solutions

12. Test solutions of the chosen concentrations are usually prepared by dilution of a stock solution. Stock solutions should preferably be prepared by dissolving the test substance in the dilution water. As far as possible, the use of solvents, emulsifiers or dispersants should be avoided. However, such compounds may be required in some cases in order to produce a suitably concentrated stock solution. Guidance for suitable solvents, emulsifiers and dispersants is given in (4). In any case, the test substance in the test solutions should not exceed the limit of solubility in the dilution water.

13. The test should be carried out without the adjustment of pH. If the pH does not remain in the range 6-9, then a second test could be carried out, adjusting the pH of the stock solution to that of the dilution water before addition of the test substance. The pH adjustment should be made in such a way that the stock solution concentration is not changed to any significant extent and that no chemical reaction or precipitation of the test substance is caused. HCl and NaOH are preferred.

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PROCEDURE

Conditions of exposure

Test groups and controls

14. Test vessels are filled with appropriate volumes of dilution water and solutions of test substance. Ratio of air/water volume in the vessel should be identical for test and control groups. Daphnids are then placed into test vessels. At least 20 animals, preferably divided into four groups of five animals each, should be used at each test concentration and for the controls. At least 2 ml of test solution should be provided for each animal (i.e. a volume of 10 ml for five daphnids per test vessel). The test may be carried out using semi-static renewal or flow-through system when the concentration of the test substance is not stable.

15. One dilution-water control series and also, if relevant, one control series containing the solubilising agent (solvent control) at the level used in treatments must be run in addition to the treatment series.

Test concentrations

16. A range-finding test may be conducted to determine the range of concentrations for the definitive test unless information on toxicity of the test substance is available. For this purpose, the daphnids are exposed to a series of widely spaced concentrations of the test substance. Five daphnids should be exposed to each test concentration for 48 hours or less, and no replicates are necessary. The exposure period may be shortened (e.g. 24 hours or less) if data suitable for the purpose of the range-finding test can be obtained in less time.

17. At least five test concentrations should be used. They should be arranged in a geometric series with a separation factor preferably not exceeding 2.2. Justification should be provided if fewer than five concentrations are used. The highest concentration tested should preferably result in 100 per cent immobilisation, and the lowest concentration tested should preferably give no observable effect.

Incubation conditions

18. The temperature should be within the range of 18°C and 22°C, and for each single test it should be constant within $\pm 1^\circ\text{C}$. A 16-hour light and 8-hour dark cycle is recommended. Complete darkness is also acceptable, especially for test substances unstable in light.

19. The test vessels must not be aerated during the test. The test is carried out without adjustment of pH. The daphnids should not be fed during the test.

Duration

20. The test duration is 48 hours.

Observations

21. Each test vessel should be checked for immobilised daphnids at 24 and 48 hours after the beginning of the test. (see Annex 1 for definitions). In addition to immobility, any abnormal behaviour or appearance should be reported.

Analytical measurements

22. The dissolved oxygen and pH are measured at the beginning and end of the test in the control(s) and in the highest test substance concentration. The dissolved oxygen concentration in controls should be in compliance with the validity criterion (see paragraph 6). The pH should normally not vary by more than 1.5 units in any one test. The temperature is usually measured in control vessels or in ambient air and it should be recorded preferably continuously during the test or, as a minimum, at the beginning and end of the test.

23. The concentration of the test substance should be measured, as a minimum, at the highest and lowest test concentration, at the beginning and end of the test (4). It is recommended that results be based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test substance has been satisfactorily maintained within ± 20 per cent of the nominal or measured initial concentration throughout the test, then the results can be based on nominal or measured initial values.

LIMIT TEST

24. Using the procedures described in this Guideline, a limit test may be performed at 100 mg/l of test substance or up to its limit of solubility in the test medium (whichever is the lower) in order to demonstrate that the EC₅₀ is greater than this concentration. The limit test should be performed using 20 daphnids (preferably divided into four groups of five), with the same number in the control(s). If the percentage of immobilisation exceeds 10% at the end of the test, a full study should be conducted. Any observed abnormal behaviour should be recorded.

DATA AND REPORTING

Data

25. Data should be summarised in tabular form, showing for each treatment group and control, the number of daphnids used, and immobilisation at each observation. The percentages immobilised at 24 hours and 48 hours are plotted against test concentrations. Data are analysed by appropriate statistical methods (e.g. probit analysis, etc.) to calculate the slopes of the curves and the EC₅₀ with 95% confidence limits ($p = 0.95$) (7)(8).

26. Where the standard methods of calculating the EC₅₀ are not applicable to the data obtained, the highest concentration causing no immobility and the lowest concentration producing 100 per cent immobility should be used as an approximation for the EC₅₀ (this being considered the geometric mean of these two concentrations).

Test report

27. The test report must include the following:

Test substance:

- physical nature and relevant physical-chemical properties;
- chemical identification data, including purity.

Test species:

- source and species of *Daphnia*, supplier of source (if known) and the culture conditions used (including source, kind and amount of food, feeding frequency).

Test conditions:

- description of test vessels: type and volume of vessels, volume of solution, number of daphnids per test vessel, number of test vessels (replicates) per concentration;
- methods of preparation of stock and test solutions including the use of any solvent or dispersants, concentrations used;
- details of dilution water: source and water quality characteristics (pH, hardness, Ca/Mg ratio, Na/K ratio, alkalinity, conductivity, etc.); composition of reconstituted water if used;
- incubation conditions: temperature, light intensity and periodicity, dissolved oxygen, pH, etc.

Results:

- the number and percentage of daphnids that were immobilised or showed any adverse effects (including abnormal behaviour) in the controls and in each treatment group, at each observation time and a description of the nature of the effects observed;
- results and date of test performed with reference substance, if available;
- the nominal test concentrations and the result of all analyses to determine the concentration of the test substance in the test vessels; the recovery efficiency of the method and the limit of determination should also be reported;
- all physical-chemical measurements of temperature, pH and dissolved oxygen made during the test;
- the EC₅₀ at 48h for immobilisation with confidence intervals and graphs of the fitted model used for their calculation, the slopes of the dose-response curves and their standard error; statistical procedures used for determination of EC₅₀; (these data items for immobilisation at 24h should also be reported when they were measured.)
- explanation for any deviation from the Test Guideline and whether the deviation affected the test results.

LITERATURE

- (1) ISO 6341. (1996). Water quality - Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) - Acute toxicity test. Third edition, 1996.
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- (6) OECD Guidelines for the Testing of Chemicals. Guideline 211: *Daphnia magna* Reproduction Test, adopted September 1998.
- (7) Stephan C.E. (1977). Methods for calculating an LC50. In Aquatic Toxicology and Hazard Evaluation (edited by F.I. Mayer and J.L. Hamelink). ASTM STP 634 - American Society for Testing and Materials. Pp65-84
- (8) Finney D.J. (1978). Statistical Methods in Biological Assay. 3rd ed. London. Griffin, Weycombe, UK.

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ANNEX 1DEFINITIONS

In the context of this guideline, the following definitions are used:

EC₅₀ is the concentration estimated to immobilise 50 per cent of the daphnids within a stated exposure period. If another definition is used, this must be reported, together with its reference.

Immobilisation: Those animals that are not able to swim within 15 seconds, after gentle agitation of the test vessel are considered to be immobilised (even if they can still move their antennae).

ANNEX 2SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

Substance	Concentration
Particulate matter	<20 mg/l
Total organic carbon	< 2 mg/l
Unionised ammonia	< 1 µg/l
Residual chlorine	<10 µg/l
Total organophosphorus pesticides	<50 ng/l
Total organochlorine pesticides plus polychlorinated biphenyls	<50 ng/l
Total organic chlorine	<25 ng/l

ANNEX 3EXAMPLES OF SUITABLE RECONSTITUTED TEST WATERISO Test water (1)

Stock solutions (single substance)		To prepare the reconstituted water, add the following volumes of stock solutions to 1 litre water*
Substance	Amount added to 1 litre water*	
Calcium chloride $\text{CaCl}_2, 2\text{H}_2\text{O}$	11.76 g	25 ml
Magnesium sulfate $\text{MgSO}_4, 7\text{H}_2\text{O}$	4.93 g	25 ml
Sodium bicarbonate NaHCO_3	2.59 g	25 ml
Potassium chloride KCl	0.23 g	25 ml

* Water of suitable purity, for example deionised, distilled or reverse osmosis with conductivity preferably not exceeding $10 \mu\text{S}\cdot\text{cm}^{-1}$.

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ANNEX 3(Cont.)Elendt M7 and M4 mediumAcclimation to Elendt M4 and M7 medium

Some laboratories have experienced difficulty in directly transferring *Daphnia* to M4 and M7 media. However, some success has been achieved with gradual acclimation, i.e. moving from own medium to 30% Elendt, then to 60% Elendt and then to 100% Elendt. The acclimation periods may need to be as long as one month.

PreparationTrace element

Separate stock solutions (I) of individual trace elements are first prepared in water of suitable purity, for example deionised, distilled or reverse osmosis. From these different stock solutions (I) a second single stock solution (II) is prepared, which contains all trace elements (combined solution), i.e.:

Stock solution(s) I (single substance)	Amount added to water (mg/l)	Concentration (related to medium M4)	To prepare the combined stock solution II, add the following amount of stock solution I to water (ml/l)	
			M4	M7
H ₃ BO ₃	57 190	20 000-fold	1.0	0.25
MnCl ₂ •4H ₂ O	7 210	20 000-fold	1.0	0.25
LiCl	6 120	20 000-fold	1.0	0.25
RbCl	1 420	20 000-fold	1.0	0.25
SrCl ₂ •6H ₂ O	3 040	20 000-fold	1.0	0.25
NaBr	320	20 000-fold	1.0	0.25
Na ₂ MoO ₄ •2H ₂ O	1 230	20 000-fold	1.0	0.25
CuCl ₂ •2H ₂ O	335	20 000-fold	1.0	0.25
ZnCl ₂	260	20 000-fold	1.0	1.0
CoCl ₂ •6H ₂ O	200	20 000-fold	1.0	1.0
KI	65	20 000-fold	1.0	1.0
Na ₂ SeO ₃	43.8	20 000-fold	1.0	1.0
NH ₄ VO ₃	11.5	20 000-fold	1.0	1.0
Na ₂ EDTA•2H ₂ O	5 000	2 000-fold	-	-
FeSO ₄ •7H ₂ O	1991	2 000-fold	-	-
Both Na ₂ EDTA and FeSO ₄ solutions are prepared singly, poured together and autoclaved immediately. This gives:				
21 Fe-EDTA solution		1 000-fold	20.0	5.0

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M4 and M7 media

M4 and M7 media are prepared using stock solution II, the macro-nutrients and vitamin as follows:

	Amount added to water (mg/l)	Concentration (related to medium M4)	Amount of stock solution II added to prepare medium (ml/l)	
			M4	M7
Stock solution II (combined trace elements)		20-fold	50	50
Macro nutrient stock solutions (single substance)				
CaCl ₂ •2H ₂ O	293 800	1 000-fold	1.0	1.0
MgSO ₄ •7H ₂ O	246 600	2 000-fold	0.5	0.5
KCl	58 000	10 000-fold	0.1	0.1
NaHCO ₃	64 800	1 000-fold	1.0	1.0
Na ₂ SiO ₃ •9H ₂ O	50 000	5 000-fold	0.2	0.2
NaNO ₃	2 740	10 000-fold	0.1	0.1
KH ₂ PO ₄	1 430	10 000-fold	0.1	0.1
K ₂ HPO ₄	1 840	10 000-fold	0.1	0.1
Combined Vitamin stock	-	10 000-fold	0.1	0.1
The combined vitamin stock solution is prepared by adding the 3 vitamin to 1 litre water, as shown below:				
Thiamine hydrochloride	750	10 000-fold		
Cyanocobalamine (B ₁₂)	10	10 000-fold		
Biotine	7.5	10 000-fold		

The combined vitamin stock is stored frozen in small aliquots. Vitamins are added to the media shortly before use.

N.B: To avoid precipitation of salts when preparing the complete media, add the aliquots of stock solutions to about 500 – 800 ml deionised water and then fill up to 1 litre.

N.N.B: The first publication of the M4 medium can be found in Elendt, B. P. (1990). Selenium deficiency in crustacea; an ultrastructural approach to antennal damage in *Daphnia magna* Straus. *Protoplasma*, 154, 25-33.

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para 49, 50**OECD GUIDELINE FOR TESTING OF CHEMICALS**Adopted by the Council on 17th July 1992(HCA)
(No 5+1)**Fish, Acute Toxicity Test****INTRODUCTION**

1. This new version of the guideline, originally adopted in 1981 and first updated in 1984, is based on a proposal from the United Kingdom to reduce the numbers of fish in tests of acute aquatic toxicity. The proposal was discussed at a meeting of OECD experts convened at Medmenham (United Kingdom) in November 1988.
2. The main differences in comparison with the earlier versions are the reduction in group-size allowing the use of seven fish per group, the extension of the concentration range by allowing a spacing factor of 2.2 instead of 2 and the introduction of a limit test at 100 mg/l of test substance.

PRINCIPLE OF THE TEST

3. The fish are exposed to the test substance preferably for a period of 96 hours. Mortalities are recorded at 24, 48, 72 and 96 hours and the concentrations which kill 50 per cent of the fish (LC50) are determined where possible.

INFORMATION ON THE TEST SUBSTANCE

4. It is necessary to know the water solubility of the substance under the conditions of the test. A reliable analytical method for the quantification of the substance in the test solutions must also be available.
5. Useful information includes the structural formula, purity of the substance, stability in water and light, pK_a , P_{ow} , vapour pressure and results of a test for ready biodegradability (see Guideline 301). Solubility and vapour pressure can be used to calculate Henry's constant which will indicate if losses of the test substance may occur.

VALIDITY OF THE TEST

6. For a test to be valid the following conditions should be fulfilled:
the mortality in the control(s) should not exceed 10 per cent (or one fish if less than ten are used) at the end of the test;

- constant conditions should be maintained as far as possible throughout the test and, if necessary, semi-static or flow-through procedures should be used (see Annex 1 for definitions);
- the dissolved oxygen concentration must have been at least 60 per cent of the air saturation value throughout the test;
- there must be evidence that the concentration of the substance being tested has been satisfactorily maintained, and preferably it should be at least 80 per cent of the nominal concentration throughout the test. If the deviation from the nominal concentration is greater than 20 per cent, results should be based on the measured concentration.

DESCRIPTION OF THE METHOD

Apparatus

7. Normal laboratory equipment and especially the following is necessary :
- (a) oxygen meter;
 - (b) equipment for determination of hardness of water;
 - (c) adequate apparatus for temperature control;
 - (d) tanks made of chemically inert material and of a suitable capacity in relation to the recommended loading.

Selection of species

8. One or more species may be used, the choice being at the discretion of the testing laboratory. It is suggested that the species used be selected on the basis of such important practical criteria as, for example, their ready availability throughout the year, ease of maintenance, convenience for testing and any relevant economic, biological or ecological factors. The fish should be in good health and free from any apparent malformation.

9. Examples of fish recommended for testing are given in the Table. The fish mentioned in the Table are easy to rear and/or widely available throughout the year. They can be bred and cultivated either in fish farms or in the laboratory, under disease- and parasite-controlled conditions, so that the test fish will be healthy and of known parentage. These fish are available in many parts of the world. If other species fulfilling the above criteria are used, the test method should be adapted in such a way as to provide suitable test conditions.

Holding of fish

10. All fish must be obtained and held in the laboratory for at least 12 days before they are used for testing. They must be held in water of the quality to be used in the test for at least seven days immediately before testing and under the following conditions:

- Light: 12 to 16 hours photoperiod daily;
- Temperature: appropriate to the species (see Table);
- Oxygen
concentration: at least 80 per cent of air saturation value;

Feeding: three times per week or daily until 24 hours before the test is started.

11. Following a 48-hour settling-in period, mortalities are recorded and the following criteria applied:

- mortalities of greater than 10 per cent of population in seven days: rejection of entire batch;
- mortalities of between 5 and 10 per cent of population: acclimatisation continued for seven additional days;
- mortalities of less than 5 per cent of population: acceptance of batch.

Water

12. Good quality natural water or reconstituted water (see Annex 2) is preferred, although drinking water (dechlorinated if necessary) may also be used. Waters with total hardness of between 10 and 250 mg CaCO₃ per liter, and with a pH 6.0 to 8.5 are preferable. The reagents used for the preparation of reconstituted water should be of analytical grade and the deionised or distilled water should be of conductivity equal to or less than 10 µS cm⁻¹.

Test solutions

13. Test solutions of the chosen concentrations are prepared by dilution of a stock solution. Stock solutions of substances of low water solubility may be prepared by ultrasonic dispersion or other suitable physical means. If necessary, vehicles such as organic solvents, emulsifiers or dispersants of low toxicity to fish may be used. When such vehicles are used an additional control should be exposed to the same concentration of the vehicle as that used in the most concentrated solution of the test substance. The concentration of organic solvents, emulsifiers or dispersants should not exceed 100 mg/l.

14. The test should be carried out without adjustment of pH. If there is evidence of marked change in the pH of the tank water after addition of the test substance, it is advisable that the test be repeated, adjusting the pH of the stock solution to that of the tank water before addition of the test substance. This pH adjustment should be made in such a way that the stock solution concentration is not changed to any significant extent and that no chemical reaction or precipitation of the test substance is caused. HCl and NaOH are preferred.

PROCEDURE

Conditions of exposure

15. Duration: preferably 96 hours.
- Loading: maximum loading of 1.0 g fish/litre for static and semi-static tests is recommended; for flow-through systems higher loading can be accepted.
- Light: 12 to 16 hours photoperiod daily.
- Temperature: appropriate to the species (see Table) and constant within a range of 2°C.

Oxygen concentration: not less than 60 per cent of the air saturation value. Aeration can be used provided that it does not lead to a significant loss of test substance.

Feeding: none.

Disturbance: disturbances that may change the behaviour of the fish should be avoided.

Number of fish

16. At least 7 fish must be used at each test concentration and in the controls.

Test concentrations

17. At least five concentrations in a geometric series with a factor preferably not exceeding 2.2. A range-finding test properly conducted before the definitive test enables the choice of the appropriate concentration range.

Controls

18. One blank and, if relevant, one control containing the solubilising agent are run in addition to the test series.

Observations

19. The fish are inspected at least after 24, 48, 72 and 96 hours. Fish are considered dead if there is no visible movement (e.g. gill movements) and if touching of the caudal peduncle produces no reaction. Dead fish are removed when observed and mortalities are recorded. Observations at three and six hours after the start of the test are desirable. Records are kept of visible abnormalities (e.g. loss of equilibrium, swimming behaviour, respiratory function, pigmentation, etc.). Measurement of pH, dissolved oxygen and temperature should be carried out at least daily.

LIMIT TEST

20. Using the procedures described in this Guideline, a limit test may be performed at 100 mg(active ingredient)/l in order to demonstrate that the LC50 is greater than this concentration. The limit test should be performed using a minimum of 7 fish, with the same number in the control(s). (Binomial theory dictates that when 10 fish are used with zero mortality, there is a 99.9 % confidence that the LC50 is greater than 100 mg/l. With 7, 8 or 9 fish, the absence of mortality provides at least 99% confidence that the LC50 is greater than the concentration used in the limit test.) If any mortalities occur, a full study should be conducted. If sublethal effects are observed, these should be recorded.

DATA AND REPORTING

Treatment of results

21. The cumulative percentage mortality for each exposure period is plotted against concentration on logarithmic probability paper. Normal statistical procedures are then employed to calculate the LC50 for the appropriate exposure period. Confidence limits ($p = 0.95$) for the calculated LC50 values are determined using standard procedures (1)(2)(3)(4)(5).

22. Where the data obtained are inadequate for the use of standard methods of calculating the LC50, the highest concentration causing no mortality and the lowest concentration producing 100 per cent mortality should be used as an approximation for the LC50 (this being considered the geometric mean of these two concentrations).

Test report

23. The test report must include the following information:

Test substance:

- physical nature and, where relevant, physicochemical properties;
- identification data.

Test fish:

- scientific name, strain, size, supplier, any pretreatment, etc.

Test conditions:

- test procedure used (e.g. static, semi-static, flow-through; aeration; fish loading; etc.);
- water quality characteristics (pH, hardness, temperature);
- dissolved oxygen concentration, pH values and temperature of the test solutions at 24 hour intervals (in semi-static systems the pH should be measured prior to and after water renewal);
- methods of preparation of stock and test solutions;
- concentrations used;
- information on concentrations of the test substance in the test solutions;
- number of fish in each test solution.

Results:

- maximum concentration causing no mortality within the period of the test;
- minimum concentration causing 100 per cent mortality within the period of the test;
- cumulative mortality at each concentration at the recommended observation times;
- LC50 values, with 95 per cent confidence limits, at each of the recommended observation times, if possible;
- graph of the concentration-mortality curve at the end of the test;
- statistical procedures used for determining the LC50 values;
- mortality in the controls;
- incidents in the course of the test which might have influenced the results;
- abnormal responses of the fish.

Discussion of the results.

TABLE: FISH SPECIES RECOMMENDED FOR TESTING

Recommended species	Recommended test temperature range (°C)	Recommended total length of test fish (cm) ¹
<u>Brachydanio rerio</u> (Teleostei, Cyprinidae) (Hamilton-Buchanan) Zebra-fish	21 - 25	2.0 ± 1.0
<u>Pimephales promelas</u> (Teleostei, Cyprinidae) (Rafinesque) Fathead Minnow	21 - 25	2.0 ± 1.0
<u>Cyprinus carpio</u> (Teleostei, Cyprinidae) (Linnaeus) Common carp	20 - 24	3.0 ± 1.0
<u>Oryzias latipes</u> (Teleostei, Cyprinodontidae) (Temminck and Schlegel) Ricefish	21 - 25	2.0 ± 1.0
<u>Poecilia reticulata</u> (Teleostei, Poeciliidae) (Peters) Guppy	21 - 25	2.0 ± 1.0
<u>Lepomis macrochirus</u> (Teleostei, Centrarchidae) (Rafinesque) Bluegill	21 - 25	2.0 ± 1.0
<u>Oncorhynchus mykiss</u> (Teleostei, Salmonidae) (Walbaum) Rainbow trout	13 - 17	5.0 ± 1.0

¹ If fish of sizes other than those recommended are used, this should be reported together with the rationale.

LITERATURE

- (1) Litchfield J.T. and Wilcoxon F. (1949). A simplified method of evaluating dose-effect experiments. *J. Pharmacol and Exper. Ther.*, 96, 99-113.
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ANNEX 1DEFINITIONS

Static test is a test with aquatic organisms in which no flow of test solution occurs. Solutions remain unchanged throughout the duration of the test.

Semi-static test is a test without flow of solution, but with occasional batchwise renewal of the test solution after prolonged periods (e.g. 24 hours).

Flow-through test is a test in which solutions are automatically and continually renewed in the test chambers, the displaced solutions running to waste.

LC50 in this Test Guideline is the median lethal concentration, i.e. that concentration of the test substance in water which kills 50 per cent of a test batch of fish within a particular period of exposure (which must be stated).

ANNEX 2EXAMPLE OF A SUITABLE RECONSTITUTED WATER (ISO 6341-1982)

- (a) Calcium chloride solution
Dissolve 11.76 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in deionised water; make up to 1 litre with deionised water
- (b) Magnesium sulphate solution
Dissolve 4.93 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in deionised water; make up to 1 litre with deionised water
- (c) Sodium bicarbonate solution
Dissolve 2.59 g NaHCO_3 in deionised water; make up to 1 litre with deionised water
- (d) Potassium chloride solution
Dissolve 0.23 g KCl in deionised water; make up to 1 litre with deionised water

All chemicals must be of analytical grade.

The conductivity of the distilled or deionised water should not exceed $10 \mu\text{Scm}^{-1}$.

25 ml each of solutions (a) to (d) are mixed and the total volume made up to 1 litre with deionised water. The sum of the calcium and magnesium ions in this solution is 2.5 mmol/l. The proportion Ca:Mg ions is 4:1 and Na:K ions 10:1. The acid capacity $K_{\text{S4.3}}$ of this solution is 0.8 mmol/l.

Aerate the dilution water until oxygen saturation is achieved, then store it for about two days without further aeration before use.

OECD GUIDELINES FOR THE TESTING OF CHEMICALS**Sediment-Water Chironomid Toxicity Test Using Spiked Sediment****INTRODUCTION**

1. This Test Guideline is designed to assess the effects of prolonged exposure of chemicals to the sediment-dwelling larvae of the freshwater dipteran *Chironomus* sp. It is based on existing toxicity test protocols for *Chironomus riparius* and *Chironomus tentans* which have been developed in Europe (1)(2)(3) and North America (4)(5)(6)(7)(8) and ring-tested (1)(6)(9). Other well documented chironomid species may also be used, for example *Chironomus yoshimatsui* (10)(11).

2. The exposure scenario used in this guideline is spiking of sediment with the test substance. The selection of the appropriate exposure scenario depends on the intended application of the test. The scenario of spiking sediment is intended to simulate accumulated levels of chemicals persisting in the sediment. This exposure system involves spiking sediment of a sediment-water test system.

3. Substances that need to be tested towards sediment-dwelling organisms usually persist in this compartment over long time periods. The sediment-dwelling organisms may be exposed via a number of routes. The relative importance of each exposure route, and the time taken for each to contribute to the overall toxic effects, is dependent on the physical-chemical properties of the chemical concerned. For strongly adsorbing substances (e.g. with $\log K_{ow} > 5$) or for substances covalently binding to sediment, ingestion of contaminated food may be a significant exposure route. In order not to underestimate the toxicity of highly lipophilic substances, the use of food added to the sediment before application of the test substance may be considered. In order to take all potential routes of exposure into account the focus of this Guideline is on long-term exposure. The test duration is in the range of 20 - 28 days for *C. riparius* and *C. yoshimatsui*, and 28 - 65 days for *C. tentans*. If short-term data are required for a specific purpose, for example to investigate the effects of unstable chemical, additional replicates may be removed after a ten-day period.

4. The measured endpoints are the total number of adults emerged and the time to emergence. It is recommended that measurements of larval survival and growth should only be made after a ten-day period if additional short-term data are required, using additional replicates as appropriate.

5. The use of formulated sediment is recommended. Formulated sediment has several advantages over natural sediments:

- the experimental variability is reduced because it forms a reproducible "standardised matrix" and the need to find uncontaminated and clean sediment sources is eliminated;
- the tests can be initiated at any time without encountering seasonal variability in the test sediment and there is no need to pre-treat the sediment to remove indigenous fauna; the use of formulated sediment also reduces the cost associated with the field collection of sufficient amounts of sediment for routine testing;
- the use of formulated sediment allows for comparisons of toxicity and ranking substances accordingly.

6. Definitions used are given in Annex 1.

PRINCIPLE OF THE TEST

7. First instar chironomid larvae are exposed to a concentration range of the test chemical in sediment - water systems. The test substance is spiked into the sediment and first instar larvae are subsequently introduced into test beakers in which the sediment and water concentrations have been stabilised. Chironomid emergence and development rate is measured at the end of the test. Larval survival and weight may also be measured after 10 days if required (using additional replicates as appropriate). These data are analysed either by using a regression model in order to estimate the concentration that would cause x % reduction in emergence or larval survival or growth (e.g. EC₁₅, EC₅₀ etc.), or by using statistical hypothesis testing to determine a NOEC/LOEC. The latter requires comparison of effect values with control values using statistical tests.

INFORMATION ON THE TEST SUBSTANCE

8. The water solubility of the test substance, its vapour pressure, measured or calculated partitioning into sediment and stability in water and sediment should be known. A reliable analytical method for the quantification of the test substance in overlying water, pore water and sediment with known and reported accuracy and limit of detection should be available. Useful information includes the structural formula and purity of the test substance. Chemical fate of the test substance (e.g. dissipation, abiotic and biotic degradation, etc.) also is useful information. Further guidance for testing substances with physical-chemical properties that make them difficult to perform the test is provided in (12)

REFERENCE SUBSTANCES

9. Reference substances may be tested periodically as a means of assuring that the test protocol and test conditions are reliable. Examples of reference toxicants used successfully in ring-tests and validation studies are: lindane, trifluralin, pentachlorophenol, cadmium chloride and potassium chloride (1)(2)(5)(6)(13).

VALIDITY OF THE TEST

10. For the test to be valid the following conditions apply:
- the emergence in the controls must be at least 70% at the end of the test (1)(6);
 - *C. riparius* and *C. yoshimatsui* emergence to adults from control vessels should occur between 12 and 23 days after their insertion into the vessels; for *C. tentans*, a period of 20 to 65 days is necessary.
 - at the end of the test, pH and the dissolved oxygen concentration should be measured in each vessel. The oxygen concentration should be at least 60 per cent of the air saturation value (ASV) at the temperature used, and the pH of overlying water should be in the 6-9 range in all test vessels;
 - the water temperature should not differ by more than ± 1.0 °C. The water temperature could be controlled by isothermal room and in that case the room temperature should be confirmed in an appropriate time intervals.

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DESCRIPTION OF THE METHODTest vessels

11. The study is conducted in glass 600 ml beakers measuring 8 cm in diameter. Other vessels are suitable, but they should guarantee a suitable depth of overlying water and sediment. The sediment surface should be sufficient enough to provide 2 to 3 cm² per larvae. The ratio of the depth of the sediment layer to the depth of the overlying water should be 1:4. Test vessels and other apparatus that will come into contact with the test system should be made entirely of glass or other chemically inert material (e.g. Teflon).

Selection of species

12. The species to be used in the test is preferably *Chironomus riparius*. *Chironomus tentans* is also suitable but more difficult to handle and requires a longer test period. *Chironomus yohimatsui* may also be used. Details of culture methods are given in Annex 2 for *Chironomus riparius*. Information on culture conditions is also available for other species, i.e. *Chironomus tentans* (4) and *Chironomus yohimatsui* (11). Identification of species must be confirmed before testing but is not required prior to every test if organisms come from an in-house culture.

Sediment

13. Formulated sediment (also called reconstituted, artificial or synthetic sediment) should preferably be used. However, if natural sediment is used, it should be characterised (at least pH, organic carbon content, determination of other parameters such as C/N ratio and granulometry are also recommended), and it should be free from any contamination and other organisms that might compete with, or consume the chironomids. It is also recommended that, before it is used in a chironomid toxicity test, the natural sediment be conditioned for seven days under the same conditions which prevail in the subsequent test. The following formulated sediment, based on the artificial soil used in Guideline 207 (14), is recommended for use in this test (1)(15)(16):

- (a) 4-5 % (dry weight) peat: as close to pH 5.5 to 6.0 as possible; it is important to use peat in powder form, finely ground (particle size ≤ 1 mm) and only air dried.
- (b) 20 % (dry weight) kaolin clay (kaolinite content preferably above 30 %).
- (c) 75-76 % (dry weight) quartz sand (fine sand should predominate with more than 50 per cent of the particles between 50 and 200 μ m).
- (d) Deionised water is added to obtain a moisture content of the final mixture in a range of 30-50 %.
- (e) Calcium carbonate of chemically pure quality (CaCO₃) is added to adjust the pH of the final mixture of the sediment to 7.0 ± 0.5 . Organic carbon content of the final mixture should be 2 % (± 0.5 %) and is to be adjusted by the use of appropriate amounts of peat and sand, according to (a) and (c).

14. The source of peat, kaolin clay and sand should be known. The sediment components should be checked for the absence of chemical contamination (e.g. heavy metals, organochlorine compounds, organophosphorous compounds, etc.). An example for the preparation of the formulated sediment is described in Annex 3. Mixing of dry constituents is also acceptable if it is demonstrated that after addition of overlying water a separation of sediment constituents (e.g. floating of peat particles) does not occur, and that the peat or the sediment is sufficiently conditioned.

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Water

15. Any water which conforms to the chemical characteristics of acceptable dilution water as listed in Annexes 2 and 4 is suitable as test water. Any suitable water, natural water (surface or ground water), reconstituted water (see Annex 2) or dechlorinated tap water are acceptable as culturing water and test water if chironomids will survive in it for the duration of the culturing and testing without showing signs of stress. At the start of the test, the pH of the test water should be between 6 and 9 and the total hardness not higher than 400 mg/l as CaCO₃. However, if there is an interaction suspected between hardness ions and the test substance, lower hardness water should be used (and thus, Elendt Medium M4 must not be used in this situation). The same type of water should be used throughout the whole study. The water quality characteristics listed in Annex 4 should be measured at least twice a year or when it is suspected that these characteristics may have changed significantly.

Stock solutions - Spiked sediments

16. Spiked sediments of the chosen concentration are usually prepared by addition of a solution of the test substance directly to the sediment. A stock solution of the test substance dissolved in deionised water is mixed with the formulated sediment by rolling mill, feed mixer or hand mixing. If poorly soluble in water, the test substance can be dissolved in as small a volume as possible of a suitable organic solvent (e.g. hexane, acetone or chloroform). This solution is then mixed with 10 g of fine quartz sand for one test vessel. The solvent is allowed to evaporate and it has to be totally removed from sand; the sand is then mixed with the suitable amount of sediment per test beaker. Only agents which volatilise readily can be used to solubilise, disperse or emulsify the test substance. It should be born in mind that the sand provided by the test substance and sand mixture, has to be taken into account when preparing the sediment (i.e. the sediment should thus be prepared with less sand). Care should be taken to ensure that the test substance added to sediment is thoroughly and evenly distributed within the sediment. If necessary, subsamples can be analysed to determine degree of homogeneity.

TEST DESIGN

17. The test design relates to the selection of the number and spacing of the test concentrations, the number of vessels at each concentration and the number of larvae per vessel. Designs for EC point estimation, for estimation of NOEC, and for conducting a limit test are described.

Design for analysis by regression

18. The effect concentration (e.g. EC₁₅, EC₅₀) and the concentration range, over which the effect of the test substance is of interest, should be spanned by the concentrations included in the test. Generally, the accuracy and especially validity, with which estimates of effect concentrations (EC_x) can be made, is improved when the effect concentration is within the range of concentrations tested. Extrapolating much below the lowest positive concentration or above the highest concentration should be avoided. A preliminary range-finding test is helpful for selecting the range of concentrations to be used (see paragraph 27).

19. If the EC_x is to be estimated, at least five concentrations and three replicates for each concentration should be tested. In any case, it is advisable that sufficient test concentrations are used to allow good model estimation. The factor between concentrations should not be greater than two (an exception could be made in cases when the dose response curve has a shallow slope). The number of replicates at each treatment can be reduced if the number of test concentrations with different responses is

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increased. Increasing the number of replicates or reducing the size of the test concentration intervals tends to lead to narrower confidence intervals for the test. Additional replicates are required if 10-day larval survival and growth are to be estimated.

Design for estimation of a NOEC/LOEC

20. If the LOEC or NOEC are to be estimated, five test concentrations with at least four replicates should be used and the factor between concentrations should not be greater than two. The number of replicates should be sufficient to ensure adequate statistical power to detect a 20 % difference from the control at the 5% level of significance ($p = 0.05$). With the development rate, an Analysis of Variance (ANOVA) is usually appropriate, such as Dunnett-test and Williams-test (17)(18)(19)(20). In the emergence ratio the Cochran-Armitage, Fisher's exact (with Bonferroni correction), or Mantel-Haentzel tests may be used.

Limit test

21. A limit test may be performed (one test concentration and control) if no effects were seen in the preliminary range-finding test. The purpose of the limit test is to perform a test at a concentration sufficiently high to enable decision makers to exclude possible toxic effects of the substance, and the limit is set at a concentration which is not expected to appear in any situation. A concentration of 1000 mg/kg (dry weight) is recommended. Usually, at least six replicates for both the treatment and control are necessary. Adequate statistical power to detect a 20 % difference from the control at the 5 % level of significance ($p = 0.05$) should be demonstrated. With metric response (development rate and weight), the t-test is a suitable statistical method if data meet the requirements of this test (normality, homogeneous variances). The unequal-variance t-test or a non parametric test, such as the Wilcoxon-Mann-Whitney test may be used, if these requirements are not fulfilled. With the emergence ratio, the Fisher exact test is appropriate.

PROCEDURE

Conditions of exposure

Preparation of spiked sediment - water system

22. The spiking procedure described in OECD Guideline 207: Earthworm, Acute Toxicity Test is recommended for application of the test substance (14). The spiked sediments are placed in the vessels and overlying water is added to produce a sediment-water volume ratio of 1:4 (see paragraphs 11 and 15). The depth of the sediment layer should be in the range of 1.5–3 cm. To avoid separation of sediment ingredients and re-suspension of fine material during addition of test water in the water column, the sediment can be covered with a plastic disc while water is poured onto it, and the disc removed immediately afterwards. Other devices may also be appropriate.

23. The test vessels should be covered (e.g. by glass plates). If necessary, during the study the water levels will be topped to the original volume in order to compensate for water evaporation. This should be performed using distilled or deionised water to prevent build-up of salts.

Stabilisation

24. Once the spiked sediment with overlying water has been prepared, it is desirable to allow partitioning of the test substance from the aqueous phase to the sediment (3)(4)(6)(13). This should

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preferably be done under the conditions of temperature and aeration used in the test. Appropriate equilibration time is sediment and chemical specific, and can be in the order of hours to days and in rare cases up to several weeks (4-5 weeks). As this would leave time for degradation of many chemicals, equilibrium is not awaited but an equilibration period of 48 hours is recommended. At the end of this further equilibration period, the concentration of the test substance should be measured in the overlying water, the pore water and the sediment, at least at the highest concentration and a lower one (see paragraph 38). These analytical determinations of the test substance allow for calculation of mass balance and expression of results based on measured concentrations.

Addition of test organisms

25. Four to five days before adding the test organisms to the test vessels, egg masses should be taken from the cultures and placed in small vessels in culture medium. Aged medium from the stock culture or freshly prepared medium may be used. If the latter is used, a small amount of food, for example green algae and/or a few droplets of filtrate from a finely ground suspension of flaked fish food should be added to the culture medium (see Annex 2). Only freshly laid egg masses should be used. Normally, the larvae begin to hatch a couple of days after the eggs are laid (2 to 3 days for *Chironomus riparius* at 20 °C and 1 to 4 days for *Chironomus tentans* at 23 °C and *Chironomus yoshimatsu* at 25 °C) and larval growth occurs in four instars, each of 4-8 days duration. First instar larvae (2-3 or 1-4 days post hatching) should be used in the test. The instar of midges can possibly be checked using head capsule width (6).

26. Twenty first instar larvae are allocated randomly to each test vessel containing the spiked sediment and water, using a blunt pipette. Aeration of the water has to be stopped while adding the larvae to test vessels and remain so for another 24 hours after addition of larvae (see paragraphs 25 and 32). According to the test design used (see paragraphs 19 and 20), the number of larvae used per concentration is at least 60 for the EC point estimation and 80 for determination of NOEC.

Test concentrations

27. A range-finding test may be helpful to determine the range of concentrations for the definitive test. For this purpose a series of widely spaced concentrations of the test substance are used. In order to provide the same density of surface per chironomids, which is to be used for the definitive test, chironomids are exposed to each concentration of the test substance for a period which allows estimation of appropriate test concentrations, and no replicates are required.

28. The test concentrations for the definitive test are decided based on the result of the range-finding test. At least five concentrations should be used and selected as described in paragraphs 18 to 20.

Controls

29. Control vessels without any test chemical but including sediment should be included in the test with the appropriate number of replicates (see paragraphs 19-20). If a solvent has been used for application of test substance (see paragraph 16), a sediment solvent control should be added.

Test system

30. Static systems are used. Semi-static or flow-through systems with intermittent or continuous renewal of overlying water might be used in exceptional cases as for instance if water quality specifications become inappropriate for the test organism or affect chemical equilibrium (e.g. dissolved oxygen levels fall too low, the concentration of excretory products rises too high or minerals leach from sediment and affect

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pH and/or water hardness). However, other methods for ameliorating the quality of overlying water, such as aeration, will normally suffice and be preferable.

Food

31. It is necessary to feed the larvae, preferably daily or at least three times per week. Fish-food (a suspension in water or finely ground food, for example Tetra-Min or Tetra-Phyll; see details in Annex 2) in the amount of 0.25-0.5 mg (0.35-0.5 mg for *C. yoshimatsui*) per larvae per day seems adequate for young larvae for the first 10 days. Slightly more food may be necessary for older larvae: 0.5- 1 mg per larvae per day should be sufficient for the rest of the test. The food ration should be reduced in all treatments and control if fungal growth is seen or if mortality is observed in controls. If fungal development cannot be stopped the test is to be repeated. When testing strongly adsorbing substances (e.g. with $\log K_{ow} > 5$), or substances covalently binding to sediment, the amount of food necessary to ensure survival and natural growth of the organisms may be added to the formulated sediment before the stabilisation period. For this, plant material must be used instead of fish food, for example the addition of 0.5% (dry weight) finely ground leaves of stinging nettle (*Urtica dioeca*), mulberry (*Morus alba*), white clover (*Trifolium repens*), spinach (*Spinacia oleracea*) or of other plant material (*Cerophyl* or alpha-cellulose) may be used.

Incubation conditions

32. Gentle aeration of the overlying water in test vessels is supplied preferably 24 hours after addition of the larvae and is pursued throughout the test (care should be taken that dissolved oxygen concentration does not fall below 60 per cent of ASV). Aeration is provided through a glass Pasteur pipette fixed 2-3 cm above the sediment layer (i.e. one or few bubbles/sec). When testing volatile chemicals, consideration may be given not to aerate the sediment-water system.

33. The test is conducted at a constant temperature of 20°C ($\pm 2^\circ\text{C}$). For *C. tentans* and *C. yoshimatsui* recommended temperatures are 23 °C and 25 °C ($\pm 2^\circ\text{C}$), respectively. A 16 hours photoperiod is used and the light intensity should be 500 to 1000 lux.

Exposure duration

34. The exposure commences with the addition of larvae to the spiked and control vessels. The maximum exposure duration is 28 days for *C. riparius* and *C. yoshimatsui*, and 65 days for *C. tentans*. If midges emerge earlier, the test can be terminated after a minimum of five days after emergence of the last adult in the control.

Observations**Emergence**

35. The development time and the total number of fully emerged male and female midges are determined. Males are easily identified by their plumose antennae.

36. The test vessels should be observed at least three times per week to make visual assessment of any abnormal behaviour (for example leaving sediment, unusual swimming), compared with the control. During the period of expected emergence a daily count of emerged midges is necessary. The sex and number of fully emerged midges are recorded daily. After identification the midges are removed from the vessels. Any egg masses deposited prior to the termination of the test should be recorded and then

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removed to prevent re-introduction of larvae into the sediment. The number of visible pupae that have failed to emerge is also recorded. Guidance on measurement of emergence is provided in Annex 5.

Growth and survival

37. If data on 10-day larval survival and growth are to be provided, additional test vessels should be included at the start, so that they may be used subsequently. The sediment from these additional vessels is sieved using a 250 µm sieve to retain the larvae. Criteria for death are immobility or lack of reaction to a mechanical stimulus. Larvae not recovered should also be counted as dead (larvae which have died at the beginning of the test may have been degraded by microbes). The (ash free) dry weight of the surviving larvae per test vessel is determined and the mean individual dry weight per vessel calculated. It is useful to determine which instar the surviving larvae belong to; for that measurement of the width of the head capsule of each individual can be used.

Analytical measurements

Concentration of the test substance

38. Prior to test commencement (i.e. addition of larvae), samples of bulk sediment are removed from at least one vessel per treatment for the analytical determination of the test substance concentration in the sediment. It is recommended that, as a minimum, samples of the overlying water, the pore water and the sediment be analysed at the start (see paragraph 24) and at the end of the test, at the highest concentration and a lower one. These determinations of test substance concentration inform about the behaviour/partitioning of the tested chemical in the water-sediment system.

39. When intermediate measurements are made (for example at day 7) and if the analysis needs large samples which cannot be taken from test vessels without influencing the test system, analytical determinations should be performed on samples from additional test vessels treated in the same way (including the presence of test organisms) but not used for biological observations.

40. Centrifugation at, for example, 10,000 g and 4°C for 30 min. is the recommended procedure to isolate interstitial water. However, if the test substance is demonstrated not to adsorb to filters, filtration may also be acceptable. In some cases it might not be possible to analyse concentrations in the pore water as the sample size is too small.

Physical-chemical parameters

41. The pH and temperature of the test vessels should be measured in an appropriate manner (see paragraph 10). Hardness and ammonia should be measured in the controls and one test vessel at the highest concentration at the start and the end of the test.

DATA AND REPORTING**Treatment of results**

42. The purpose of this test is to determine the effect of the test substance on the development rate and the total number of fully emerged male and female midges, or in the case of the 10-day test effects on survival and weight of the larvae. If there are no indications of statistically different sensitivities of sexes, male and female results may be pooled for statistical analyses. The sensitivity differences between sexes can be statistically judged by, for example, a χ^2 -r $\times$ 2 table test. Larval survival and mean individual dry weight per vessel must be determined after 10 days where required.

43. Effect concentrations expressed and based on dry weight, are calculated preferably based on measured sediment concentrations at the beginning of the test (see paragraph 38).

44. To compute a point estimate for the EC_{50} or any other EC_x , the per-vessel statistics may be used as true replicates. In calculating a confidence interval for any EC_x the variability among vessels should be taken into account, or it should be shown that this variability is so small that it can be ignored. When the model is fitted by Least Squares, a transformation should be applied to the per-vessel statistics in order to improve the homogeneity of variance. However, EC_x values should be calculated after the response is transformed back to the original value.

45. When the statistical analysis aims at determining the NOEC/LOEC by hypothesis testing, the variability among vessels needs to be taken into account, for example by a nested ANOVA. Alternatively, more robust tests (21) can be appropriate in situations where there are violations of the usual ANOVA assumptions.

Emergence ratio

46. Emergence ratios are quantal data, and can be analyzed by the Cochran-Armitage test applied in step-down manner where a monotonic dose-response is expected and these data are consistent with this expectation. If not, a Fisher's exact or Mantel-Haentzel test with Bonferroni-Holm adjusted p-values can be used. If there is evidence of greater variability between replicates within the same concentration than a binomial distribution would indicate (often referenced as "extra-binomial" variation), then a robust Cochran-Armitage or Fisher exact test such as proposed in (21), should be used.

The sum of midges emerged per vessel, n_e , is determined and divided by the number of larvae introduced, n_a :

$$ER = \frac{n_e}{n_a}$$

where:

ER = emergence ratio
 n_e = number of midges emerged per vessel
 n_a = number of larvae introduced per vessel

47. An alternative that is most appropriate for large sample sizes, when there is extra binomial variance, is to treat the emergence ratio as a continuous response and use procedures such as William's test when a monotonic dose-response is expected and is consistent with these ER data. Dunnett's test would be

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appropriate where monotonicity does not hold. A large sample size is defined here as the number emerged and the number not emerging both exceeding five, on a per replicate (vessel) basis.

48. To apply ANOVA methods, values of ER should first be transformed by the arcsin-sqrt-transformation or Tukey-Freeman transformation to obtain an approximate normal distribution and to equalise variances. The Cochran-Armitage, Fisher's exact (Bonferroni), or Mantel-Haentzel tests can be applied when using the absolute frequencies. The arcsin-sqrt transformation is applied by taking the inverse sine ($\sin^{-1}$) of the square root of ER.

49. For emergence ratios, EC_x -values are calculated using regression analysis (or e.g. probit (22), logit, Weibull, appropriate commercial software etc.). If regression analysis fails (e.g. when there are less than two partial responses), other non-parametric methods such as moving average or simple interpolation are used.

Development rate

50. The mean development time represents the mean time span between the introduction of larvae (day 0 of the test) and the emergence of the experimental cohort of midges. (For the calculation of the true development time, the age of larvae at the time of introduction should be considered). The development rate is the reciprocal of the development time (unit: 1/day) and represents that portion of larval development which takes place per day. The development rate is preferred for the evaluation of these sediment toxicity studies as its variance is lower, and it is more homogeneous and closer to normal distribution as compared to development time. Hence, powerful parametric test procedures may be used with development rate rather than with development time. For development rate as a continuous response, EC_x -values can be estimated by using regression analysis (e.g. (23), (24)).

51. For the following statistical tests, the number of midges observed on inspection day x are assumed to be emerged at the mean of the time interval between day x and day $x-1$ (l = length of the inspection interval, usually 1 day). The mean development rate per vessel ($\bar{x}$) is calculated according to:

$$\bar{x} = \sum_{i=1}^m \frac{f_i x_i}{n_e}$$

where:

- $\bar{x}$: mean development rate per vessel
- i : index of inspection interval
- m : maximum number of inspection intervals
- f_i : number of midges emerged in the inspection interval i
- n_e : total number of midges emerged at the end of experiment ($= \sum f_i$)
- x_i : development rate of the midges emerged in interval i

$$x_i = \frac{1}{\left(\text{day}_i - \frac{l_i}{2}\right)}$$

where:

- day_i : inspection day (days since application)
- l_i : length of inspection interval i (days, usually 1 day)

Test report

52. The test report must at least provide the following information:

Test substance:

- physical nature and, where relevant, physical-chemical properties (water solubility, vapour pressure, partition coefficient in soil (or in sediment if available), stability in water, etc.);
- chemical identification data (common name, chemical name, structural formula, CAS number, etc.) including purity and analytical method for quantification of test substance.

Test species:

- test animals used: species, scientific name, source of organisms and breeding conditions;
- information on handling of egg masses and larvae;
- age of test animals when inserted into test vessels.

Test conditions:

- sediment used, i.e. natural or formulated sediment;
- for natural sediment, location and description of sediment sampling site, including, if possible, contamination history; characteristics: pH, organic carbon content, C/N ratio and granulometry (if appropriate);
- preparation of the formulated sediment: ingredients and characteristics (organic carbon content, pH, moisture, etc. at the start of the test);
- preparation of the test water (if reconstituted water is used) and characteristics (oxygen concentration, pH, conductivity, hardness, etc. at the start of the test);
- depth of sediment and overlying water;
- volume of overlying and pore water; weight of wet sediment with and without pore water;
- test vessels (material and size);
- method of spiking sediment: test concentrations used, number of replicates and use of solvent if any;
- stabilisation equilibrium phase of the spiked sediment-water system: duration and conditions;
- incubation conditions: temperature, light cycle and intensity, aeration (frequency and intensity);
- detailed information on feeding including type of food, preparation, amount and feeding regime.

Results:

- the nominal test concentrations, the measured test concentrations and the results of all analyses to determine the concentration of the test substance in the test vessel;
- water quality within the test vessels, i.e. pH, temperature, dissolved oxygen, hardness and ammonia;
- replacement of evaporated test water, if any;
- number of emerged male and female midges per vessel and per day;
- number of larvae which failed to emerge as midges per vessel;
- mean individual dry weight of larvae per vessel, and per instar, if appropriate;
- percent emergence per replicate and test concentration (male and female midges pooled);
- mean development rate of fully emerged midges per replicate and treatment rate (male and female midges pooled);

- estimates of toxic endpoints, for example EC_x (and associated confidence intervals), NOEC and/or LOEC, and the statistical methods used for their determination;
- discussion of the results, including any influence on the outcome of the test resulting from deviations from this Guideline.

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ANNEX 1DEFINITIONS

For the purpose of this guideline the following definitions are used:

Formulated sediment or reconstituted, artificial or synthetic sediment, is a mixture of materials used to mimic the physical components of a natural sediment.

Overlying water is the water placed over sediment in the test vessel.

Interstitial water or pore water is the water occupying space between sediment and soil particles.

Spiked sediment is sediment to which test substance has been added.

ANNEX 2RECOMMENDATIONS FOR CULTURE OF *CHIRONOMUS RIPARIUS*

1. *Chironomus* larvae may be reared in crystallising dishes or larger containers. Fine quartz sand is spread in a thin layer of about 5 to 10 mm deep over the bottom of the container. Kieselgur (e.g. Merck, Art 8117) has also been shown to be a suitable substrate (a thinner layer of up to a very few mm is sufficient). Suitable water is then added to a depth of several cm. Water levels should be topped up as necessary to replace evaporative loss, and prevent desiccation. Water can be replaced if necessary. Gentle aeration should be provided. The larval rearing vessels should be held in a suitable cage which will prevent escape of the emerging adults. The cage should be sufficiently large to allow swarming of emerged adults, otherwise copulation may not occur (minimum is ca. 30 x 30 x 30 cm).

2. Cages should be held at room temperature or in a constant environment room at 20 ± 2 °C with a photo period of 16 hour light (intensity ca. 1000 lux), 8 hours dark. It has been reported that air humidity of less than 60 % RH can impede reproduction.

Dilution water

3. Any suitable natural or synthetic water may be used. Well water, dechlorinated tap water and artificial media (e.g. Elendt "M4" or "M7" medium, see below) are commonly used. The water has to be aerated before use. If necessary, the culture water may be renewed by pouring or siphoning the used water from culture vessels carefully without destroying the tubes of larvae.

Feeding larvae

4. *Chironomus* larvae should be fed with a fish flake food (Tetra Min[®], Tetra Phyll[®] or other similar brand of proprietary fish food), at approximately 250 mg per vessel per day. This can be given as a dry ground powder or as a suspension in water: 1.0 g of flake food is added to 20 ml of dilution water and blended to give a homogenous mix. This preparation may be fed at a rate of about 5 ml per vessel per day. (shake before use.) Older larvae may receive more.

5. Feeding is adjusted according to the water quality. If the culture medium becomes 'cloudy', the feeding should be reduced. Food additions must be carefully monitored. Too little food will cause emigration of the larvae towards the water column, and too much food will cause increased microbial activity and reduced oxygen concentrations. Both conditions can result in reduced growth rates.

6. Some green algae (e.g. *Scenedesmus subspicatus*, *Chlorella vulgaris*) cells may also be added when new culture vessels are set up.

Feeding emerged adults

7. Some experimenters have suggested that a cotton wool pad soaked in a saturated sucrose solution may serve as a food for emerged adults.

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Emergence

8. At 20 ± 2 °C adults will begin to emerge from the larval rearing vessels after approximately 13 - 15 days. Males are easily distinguished by having plumose antennae.

Egg masses

9. Once adults are present within the breeding cage, all larval rearing vessels should be checked three times weekly for deposition of the gelatinous egg masses. If present, the egg masses should be carefully removed. They should be transferred to a small dish containing a sample of the breeding water. Egg masses are used to start a new culture vessel (e.g. 2 - 4 egg masses / vessel) or are used for toxicity tests.

10. First instar larvae should hatch after 2 - 3 days.

Set-up of new culture vessels

11. Once cultures are established it should be possible to set up a fresh larval culture vessel weekly or less frequently depending on testing requirements, removing the older vessels after adult midges have emerged. Using this system a regular supply of adults will be produced with a minimum of management.

Preparation of test solutions "M4" and "M7"

12. Elendt (1990) has described the "M4" medium. The "M7" medium is prepared as the "M4" medium except for the substances indicated in Table 1, for which concentrations are four times lower in "M7" than in "M4". A publication on the "M7" medium is in preparation (Elendt, personal communication). The test solution should not be prepared according to Elendt and Bias (1990) for the concentrations of $\text{NaSiO}_3 \cdot 5 \text{H}_2\text{O}$, NaNO_3 , KH_2PO_4 and K_2HPO_4 given for the preparation of the stock solutions are not adequate.

Preparation of the "M7"-medium

13. Each stock solution (I) is prepared individually and a combined stock solution (II) is prepared from these stock solutions (I) (see Table 1). Fifty ml from the combined stock Solution (II) and the amounts of each macro nutrient stock solution which are given in Table 2 are made up to 1 litre of deionised water to prepare the "M7" medium. A vitamin stock solution is prepared by adding three vitamins to deionised water as indicated in Table 3, and 0.1 ml of the combined vitamin stock solution are added to the final "M7" medium shortly before use. (The vitamin stock solution is stored frozen in small aliquots). The medium is aerated and stabilised.

Reference

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Table 1: Stock solutions of trace elements for medium M4 and M7

Stock solutions (I)	Amount (mg) made up to 1 litre of deionised water	To prepare the combined stock solution (II): mix the following amounts (ml) of stock solutions (I) and make up to 1 litre of deionised water		Final concentrations in test solutions (mg/l)	
		M4	M7	M4	M7
H ₃ BO ₃ ⁽¹⁾	57190	1.0	0.25	2.86	0.715
MnCl ₂ • 4 H ₂ O ⁽¹⁾	7210	1.0	0.25	0.361	0.090
LiCl ⁽¹⁾	6120	1.0	0.25	0.306	0.077
RbCl ⁽¹⁾	1420	1.0	0.25	0.071	0.018
SrCl ₂ • 6 H ₂ O ⁽¹⁾	3040	1.0	0.25	0.152	0.038
NaBr ⁽¹⁾	320	1.0	0.25	0.016	0.004
Na ₂ MoO ₄ • 2 H ₂ O ⁽¹⁾	1260	1.0	0.25	0.063	0.016
CuCl ₂ • 2 H ₂ O ⁽¹⁾	335	1.0	0.25	0.017	0.004
ZnCl ₂	260	1.0	1.0	0.013	0.013
CoCl ₂ • 6 H ₂ O	200	1.0	1.0	0.010	0.010
KI	65	1.0	1.0	0.0033	0.0033
Na ₂ SeO ₃	43.8	1.0	1.0	0.0022	0.0022
NH ₄ VO ₃	11.5	1.0	1.0	0.00058	0.00058
Na ₂ EDTA • 2 H ₂ O ⁽¹⁾⁽²⁾	5000	20.0	5.0	2.5	0.625
FeSO ₄ • 7 H ₂ O ⁽¹⁾⁽²⁾	1991	20.0	5.0	1.0	0.249

(1) These substances differ in M4 and M7, as indicated above.

(2) These solutions are prepared individually, then poured together and autoclaved immediately.

Table 2: Macro nutrient stock solutions for medium M4 and M7

	Amount made up to 1 litre of deionised water (mg)	Amount of macro nutrient stock solutions added to prepare medium M4 and M7 (ml/l)	Final concentrations in test solutions M4 and M7 (mg/l)
CaCl ₂ • 2 H ₂ O	293800	1.0	293.8
MgSO ₄ • 7 H ₂ O	246600	0.5	123.3
KCl	58000	0.1	5.8
NaHCO ₃	64800	1.0	64.8
NaSiO ₃ • 9 H ₂ O	50000	0.2	10.0
NaNO ₃	2740	0.1	0.274
KH ₂ PO ₄	1430	0.1	0.143
K ₂ HPO ₄	1840	0.1	0.184

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Table 3: Vitamin stock solution for medium M4 and M7

All three vitamin solutions are combined to make a single vitamin stock solution.

	Amount made up to 1 litre of deionised water (mg)	Amount of vitamin stock solution added to prepare medium M4 and M7 (ml/l)	Final concentrations in test solutions M4 and M7 (mg/l)
Thiamine hydrochloride	750	0.1	0.075
Cyanocobalamin (B12)	10	0.1	0.0010
Biotine	7.5	0.1	0.00075

References

- Elendt, B.P. (1990): Selenium Deficiency in Crustacean. *Protoplasma* 154, 25-33
- Elendt, B.P. & W.-R. Bias (1990): Trace Nutrient Deficiency in *Daphnia magna* Cultured in Standard Medium for Toxicity Testing. Effects on the Optimization of Culture Conditions on Life History Parameters of *D. magna*. *Water Research* 24 (9), 1157-1167

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ANNEX 3PREPARATION OF FORMULATED SEDIMENTSediment composition

The composition of the formulated sediment should be as follows:

Constituent	Characteristics	% of sediment dry weight
Peat	Sphagnum moss peat, as close to pH 5.5-6.0 as possible, no visible plant remains, finely ground (particle size ≤ 1 mm) and air dried	4-5
Quartz sand	Grain size: > 50% of the particles should be in the range of 50-200 μm	75-76
Kaolinite clay	Kaolinite content $\geq 30\%$	20
Organic carbon	Adjusted by addition of peat and sand	2 (± 0.5)
Calcium carbonate	CaCO_3 , pulverised, chemically pure	0.05 – 0.1
Water	Conductivity ≤ 10 $\mu\text{S/cm}$	30 – 50

Preparation

The peat is air dried and ground to a fine powder. A suspension of the required amount of peat powder in deionised water is prepared using a high-performance homogenising device. The pH of this suspension is adjusted to 5.5 ± 0.5 with CaCO_3 . The suspension is conditioned for at least two days with gentle stirring at 20 ± 2 °C, to stabilise pH and establish a stable microbial component. pH is measured again and should be 6.0 ± 0.5 . Then the peat suspension is mixed with the other constituents (sand and kaolin clay) and deionised water to obtain an homogeneous sediment with a water content in a range of 30–50 per cent of dry weight of the sediment. The pH of the final mixture is measured once again and is adjusted to 6.5 to 7.5 with CaCO_3 if necessary. Samples of the sediment are taken to determine the dry weight and the organic carbon content. Then, before it is used in the chironomid toxicity test, it is recommended that the formulated sediment be conditioned for seven days under the same conditions which prevail in the subsequent test.

Storage

The dry constituents for preparation of the artificial sediment may be stored in a dry and cool place at room temperature. The formulated (wet) sediment should not be stored prior to its use in the test. It should be used immediately after the 7 days conditioning period that ends its preparation.

References

OECD Guideline 207. (1984). Earthworm, Acute Toxicity Test.

Meller, M., P. Egeler, J. Rombke, H. Schallnass, R. Nagel and B. Streit. (1998). Short-term Toxicity of Lindane, Hexachlorobenzene and Copper Sulfate on Tubificid Sludgeworms (*Oligochaeta*) in Artificial Media. *Ecotox. and Environ. Safety*, 39, 10-20.

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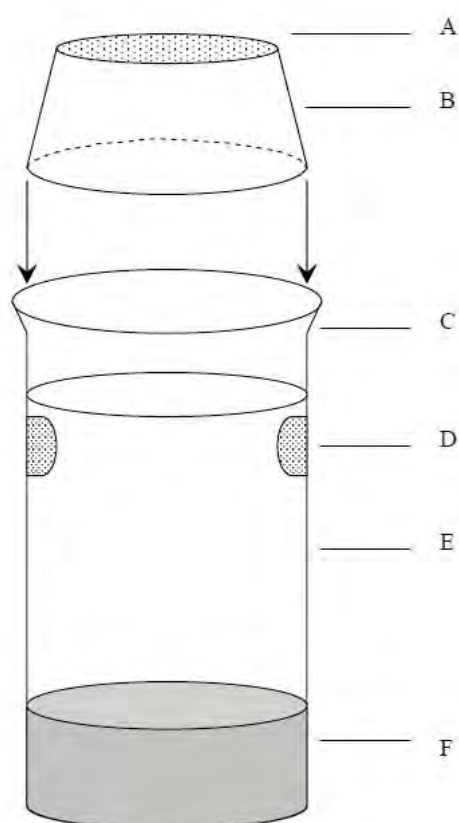
ANNEX 4CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

SUBSTANCE	CONCENTRATIONS
Particulate matter	< 20 mg/l
Total organic carbon	< 2 mg/l
Unionised ammonia	< 1 µg/l
Hardness as CaCO ₃	<400 mg/l*
Residual chlorine	< 10 µg/l
Total organophosphorus pesticides	< 50 ng/l
Total organochlorine pesticides plus polychlorinated biphenyls	< 50 ng/l
Total organic chlorine	< 25 ng/l

* However, it should be noted that if there is an interaction suspected between hardness ions and the test substance, lower hardness water should be used (and thus, Elendt Medium M4 must not be used in this situation).

ANNEX 5GUIDANCE FOR MONITORING EMERGENCE OF CHIRONOMID LARVAE

Emergence traps are placed on the test beakers. These traps are needed from day 20 to the end of the test. Example of trap used is drawn below:



A: the nylon screen
B: the inverted plastic cups
C: the lipless exposure beaker

D: the water exchange screen ports
E: water
F: sediment