OECD/OCDE TG 238
Adopted:
26 September 2014

OECD GUIDELINES FOR THE TESTING OF CHEMICALS

SEDIMENT-FREE *MYRIOPHYLLUM SPICATUM* TOXICITY TEST

INTRODUCTION

1. This Test Guideline is designed to assess the toxicity of chemicals to *Myriophyllum spicatum*, a submersed aquatic dicotyledon, a species of the water milfoils family. It is based on an ASTM existing test method (1) modified as a sediment-free test system (2) to estimate the intrinsic ecotoxicity of test chemicals (independent of the distribution-behaviour of the test chemical between water and sediment). A test system without sediment has a low analytical complexity (only in the water phase) and the results can be analysed in parallel and/or comparison with those obtained in *Lemna sp.* test (3); in addition the required sterile conditions allow to keep the effects of microorganisms and algae (compound uptake/ degradation, etc.) as low as possible. This test does not replace other aquatic toxicity tests; it should rather complement them so that a more complete aquatic plant hazard and risk assessment is possible. The test method has been validated by a ring-test (4).

2. Details of testing with renewal (semi-static) and without renewal (static) of the test solution are described. Depending on the objectives of the test and the regulatory requirements, the use of semi-static method is recommended, *e.g.* for substances that are rapidly lost from solution as a result of volatilisation, adsorption, photodegradation, hydrolysis, precipitation or biodegradation. Further guidance is given in (5). This Test Guideline applies to substances for which the test method has been validated, see details in the ring-test report (4) or to formulations, commercial products, or known mixtures; if a mixture is tested, its constituents should be as far as possible identified and quantified. The sediment-free *Myriophyllum spicatum* test method complements the water-sediment *Myriophyllum spicatum* Toxicity Test (6). Before use of the Test Guideline for the testing of a mixture intended for a regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

PRINCIPLE OF THE TEST

3. Continuously growing plant cultures of *Myriophyllum spicatum* (only in modified Andrews' medium, see Annex 2) are allowed to grow as monocultures in different concentrations of the test chemical over a period of 14 days in a sediment-free test system. The objective of the test is to quantify chemical-related effects on vegetative growth over this period based on assessments of selected measurement variables. Growth of shoot length, of lateral branches and roots as well as development of fresh and dry

weight and increase of whorls are the measurement variables. In addition, account is taken of distinctive qualitative changes in test organisms, such as disfigurement or chlorosis and necrosis indicated by yellowing or white and brown coloring. To quantify chemical-related effects, growth in the test solutions is compared with that of the controls and the concentration bringing about a specified x% inhibition of growth is determined and expressed as the EC_x; EC_x; "x" can be any value depending on the regulatory requirements, e.g. EC_{10}, EC_{20}, EC_{50}. It should be noted that estimates of EC_{10} and EC_{20} values are only reliable and appropriate in tests where coefficients of variation in control plants fall below the effect level being estimated, i.e. coefficients of variation should be <20% for robust estimation of an EC_{20}.

4. Both average specific growth rate (estimated from assessments of main shoot length and three additional measurement variables) and yield (estimated from the increase in main shoot length and three additional measurement variables) of untreated and treated plants should be determined. Specific growth rate (r) and yield (y) are subsequently used to determine the E_rC_x (e.g. E_rC_{10}, E_rC_{20}, E_rC_{50}) and E_yC_x (e.g. E_yC_{10}, E_yC_{20}, E_yC_{50}), respectively.

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

INFORMATION ON THE TEST CHEMICAL

6. An analytical method, with adequate sensitivity for quantification of the test substance(s) in the test medium, should be available. Information on the test chemical which may be useful in establishing the test conditions includes the structural formula, purity and impurities, water solubility, stability in water and light, acid dissociation constant (pK_a), partition coefficient octanol-water (K_{ow}), vapour pressure and biodegradability. Water solubility and vapour pressure can be used to calculate Henry´s Law constant, which will indicate if significant losses of the test chemical during the test period are likely. This will help indicate whether particular steps to control such losses should be taken. Where information on the solubility and stability of the test substance(s) are uncertain, it is recommended that these be assessed under the conditions of the test, *i.e.* growth medium, temperature, lighting regime to be used in the test.

7. The pH control of the test medium is particularly important, *e.g.* when testing metals or substances which are hydrolytically unstable. Further guidance for testing substances with physical-chemical properties that make them difficult to test is provided in (5).

VALIDITY OF THE TEST

8. For the test to be valid, the doubling time of main shoot length in the control must be less than 14 days. Using the media and test conditions described in this Guideline, this criterion can be attained using a static or semi-static test regime.

9. The mean coefficient of variation for yield based on measurements of shoot fresh weight (i.e. from test initiation to test termination) and the additional measurement variables (see paragraph 37) in the control cultures do not exceed 35% between replicates.

10. More than 50% of the replicates of the control group are kept sterile over the exposure period of 14 days, which means visibly free of contamination by other organisms such as algae, fungi and bacteria (clear solution). *Note*: Guidance on how to assess sterility is provided in the ring-test report (4).

REFERENCE SUBSTANCE

11. Reference substance(s), such as 3,5-dichlorophenol used in the ring test (4), may be tested as a mean of checking the test procedure; from the ring test data, the mean EC_{50}-values of 3,5-DCP for the different response variables (see paragraphs 37-41 of this Guideline) are between 3.2 mg/L and 6.9 mg/L (see ring test report for details about confidence interval for these values). It is advisable to test a reference substance at least twice a year or, where testing is carried out at a lower frequency, in parallel to the determination of the toxicity of a test chemical.

DESCRIPTION OF THE METHOD

Apparatus

12. All equipment in contact with the test media should be made of glass or other chemically inert material. Glassware used for culturing and testing purposes should be cleaned of chemical contaminants that might leach into the test medium and should be sterile. The test vessels should be long enough for the shoot in the control vessels to grow in the water phase without reaching the surface of the test medium at the end of the test. Thick-walled borosilicate glass test tubes without lip, inner diameter approximately 20 mm, length approximately 250 mm, with aluminium caps are recommended.

13. Since the modified Andrews' medium contains sucrose (which stimulates the growth of fungi and bacteria), the test solutions have to be prepared under sterile conditions. All liquids as well as equipment are sterilized before use. Sterilization is carried out via heated air treatment (210 °C) for 4 hours or autoclaving for 20 minutes at 121 °C. In addition, all flasks, dishes, bowls etc. and other equipment undergo flame treatment at a sterile workbench just prior to use.

14. The cultures and test vessels should not be kept together. This is best achieved using separate environmental growth chambers, incubators, or rooms. Illumination and temperature should be controllable and maintained at a constant level.

Test organism

15. *Myriophyllum spicatum* – a submersed aquatic dicotyledon – is a species of the water milfoils family. Between June and August, inconspicuous pink-white flowers protrude above the water surface. The plants are rooted in the ground by a system of robust rhizomes and can be found in the entire northern hemisphere in eutrophic, however non-polluted and more calciferous still waters with muddy substrate. *Myriophyllum spicatum* prefers fresh water, but is found in brackish water as well.

16. For the sediment-free toxicity test, sterile plants are required. If the testing laboratory does not have regular cultures of *Myriophyllum spicatum*, sterile plant material may be obtained from another laboratory or (unsterile) plant material might be taken from the field or provided by a commercial supplier; if plants come from the field a taxonomic verification of the species should be envisaged. If collected from the field or provided by a commercial supplier, plants should be sterilized (1) and maintained in culture in the same medium as used for testing for a minimum of eight weeks prior to use. Field sites used for collecting starting cultures have to be free of obvious sources of contamination. Great care should be taken to ensure that the correct species is obtained when collecting *Myriophyllum spicatum* from the field, especially in regions where it can hybridise with other *Myriophyllum* species. If obtained from another laboratory they should be similarly maintained for a minimum of three weeks. The source of plant material and the species used for testing should always be reported.

17. The quality and uniformity of the plants used for the test will have a significant influence on the outcome of the test and should therefore be selected with care. Young, rapidly growing plants without

visible lesions or discoloration (chlorosis) should be used. Details about preparation of the test organism are given in Annex 4.

Cultivation

18. To reduce the frequency of culture maintenance (*e.g.* when no *Myriophyllum* tests are planned for a period), cultures can be held under reduced illumination and temperature (50 μE m^{-2} s^{-1}, 20 ± 2 °C). Details of culturing are given in Annex 3.

19. At least 14 to 21 days before testing, sufficient test organisms are transferred aseptically into fresh sterile medium and cultured for 14 to 21 days under the conditions of the test as a pre culture. Details for preparation of a pre culture are given in Annex 4.

Test medium

20. Only one nutrient medium is recommended for *Myriophyllum spicatum* in a sediment-free test system, as described in Annex 2. A modification of the Andrews' medium is recommended for culturing and testing with *Myriophyllum spicatum* as described in (1). From five separately prepared nutrient stock solutions with addition of 3% sucrose the modified Andrews' medium will be arranged. Details about preparation of a pre-culture are given in Annex 2.

21. A tenfold concentrated, modified Andrews' medium is needed for obtaining the test solutions (by dilution as appropriate). The composition of this medium is given in Annex 2.

Test solutions

22. Test solutions are usually prepared by dilution of a stock solution. Stock solutions of the test chemical are normally prepared by dissolving the chemical in demineralized (*i.e.* distilled or deionized) water. The addition of the nutrients will be achieved by using the tenfold concentrated, modified Andrews' medium.

23. The stock solutions of the test chemical can be sterilized by autoclave at 121 °C for 20 minutes or by sterile filtration, provided that the sterilization technique used does not denaturize the test chemical. Test solutions can also be prepared in sterile demineralized water or medium, under sterile conditions. The thermo-stability and the adsorption on different surfaces should the taken into account in the selection of the sterilization procedure of the stock solutions of the test chemical. Because of that, it is recommended that the stock solutions be prepared under sterile conditions, *i.e.* using sterile material for dissolving the test chemical under sterile conditions (*e.g.* flame sterilization, laminar-flow hoods, etc.) into sterile water. This technique of preparation of sterile stock solutions is valid for both single test substances and mixtures/formulations.

24. The highest tested concentration of the test chemical should normally not exceed its water solubility under the test conditions. For test chemicals of low water solubility it may be necessary to prepare a concentrated stock solution or dispersion of the chemical using an organic solvent or dispersant in order to facilitate the addition of accurate quantities of the test chemical to the test medium and aid in its dispersion and dissolution. Every effort should be made to avoid the use of such materials. There should be no phytotoxicity resulting from the use of auxiliary solvents or dispersants. For example, commonly used solvents which do not cause phytotoxicity at concentrations up to 100 μL/L, include acetone and dimethylformamide. If a solvent or dispersant is used, its final concentration should be reported and kept to a minimum (≤ 100 μL/L), and all treatments and controls should contain the same concentration of solvent or dispersant. Further guidance on the use of dispersants is given in (5).

Test and control groups

25. Prior knowledge of the toxicity of the test chemical to *Myriophyllum spicatum* from a range-finding test will help in selecting suitable test concentrations. In the definitive toxicity test, there should normally be five (like in the *Lemna* growth inhibition test, TG 221) to seven test concentrations arranged in a geometric series; they should be chosen in order that the NOEC and EC_{50} values are bracketed by the concentration range (see below). Preferably the separation factor between test concentrations should not exceed 3.2; however a larger value may be used where the concentration-response curve is flat. Justification should be provided when fewer than five concentrations are used. At least five replicates should be used at each test concentration.

26. In setting the range of test concentrations (for range-finding and/or for the definitive toxicity test), the following should be considered:

- To determine an EC_x, test concentrations should bracket the EC_x value to ensure an appropriate level of confidence. For example, if estimating the EC_{50}, the highest test concentration should be greater than the EC_{50} value. If the EC_{50} value lies outside of the range of test concentrations, associated confidence intervals will be large and a proper assessment of the statistical fit of the model may not be possible.

- If the aim is to estimate the LOEC/NOEC, the lowest test concentration should be low enough so that growth is not significantly less than that of the control. In addition, the highest test concentration should be high enough so that growth is significantly lower than that in the control. If this is not the case, the test will have to be repeated using a different concentration range (unless the highest concentration is at the limit of solubility or the maximum required limit concentration, *e.g.* 100 mg/L).

27. Every test should include controls consisting of the same nutrient medium, test organism (choosing plant material as homogeneous as possible, fresh lateral branches from pre-cultures, shortened to 2.5 cm from base), environmental conditions and procedures as the test vessels but without the test chemical. If an auxiliary solvent or dispersant is used, an additional control treatment with the solvent/dispersant present at the same concentration as that in the vessels with the test chemical should be included. The number of replicate control vessels (and solvent vessels, if applicable) should be at least ten.

28. If determination of NOEC is not required, the test design may be altered to increase the number of concentrations and reduce the number of replicates per concentration. However, in any case the number of control replicates should be at least ten.

Exposure

29. Fresh lateral branches from pre-culture shortened to 2.5 cm from base are assigned randomly to the test vessels under aseptic conditions; each test vessel should contain one 2.5 cm lateral branch that should have an apical meristem on one end. The chosen plant material should be the same quality in each test vessel.

30. A randomized design for location of the test vessels in the incubator is required to minimise the influence of spatial differences in light intensity or temperature. A blocked design or random repositioning of the vessels (or repositioning more frequently) when observations are made is also required.

31. If a preliminary stability test shows that the test substance(s) concentration cannot be maintained (*i.e.* the measured concentration falls below 80% of the measured initial concentration) over the test

duration (14 days), a semi-static test regime is recommended. In this case, the plants should be exposed to freshly prepared test and control solutions on at least one occasion during the test (*e.g.* day 7). The frequency of exposure to fresh medium will depend on the stability of the test chemical; a higher frequency may be needed to maintain near-constant concentrations of highly unstable or volatile substances.

32. The exposure scenario through a foliar application (spray) is not covered in this Test Guideline.

Test conditions

33. Warm and/or cool white fluorescent lighting should be used to provide light irradiance in the range of about of 100-150 $\mu E\ m^{-2}\ s^{-1}$ when measured as a photosynthetically active radiation (400-700 nm) at points the same distance from the light source as the bottom of the test vessels (equivalent ca. 6000 to 9000 lux) and using a light-dark cycle of 16:8 h. The method of light detection and measurement, in particular the type of sensor, will affect the measured value. Spherical sensors (which respond to light from all angles above and below the plane of measurement) and "cosine" sensors (which respond to light from all angles above the plane of measurement) are preferred to unidirectional sensors, and will give higher readings for a multi-point light source of the type described here.

34. The temperature in the test vessels should be 23 ± 2 °C. Additional care is needed on pH drift in special cases such as when testing unstable substances or metals; the pH should remain in a range of 6-9. See (5) for further guidance.

Duration

35. The test is terminated 14 days after the plants are transferred into the test vessels.

Measurements and analytical determinations

36. At the start of the test, the main shoot length of test organism is 2.5 cm (see paragraph 29); it is measured with a ruler (see Annex 4) or by photography and image analysis. The main shoot length of test organism appearing normal or abnormal needs to be determined at the beginning of the test, at least once during the 14-day exposure period and at test termination. Note: As an alternative for those who do not have image analysis, if the workbench is sterilized prior to addition of plants to test vessels, a sterile ruler can also be used to measure the length of the main shoot at test initiation and during the test. Changes in plant development, *e.g.* in deformation in the shoots, appearance, indication of necrosis, chlorosis, break-up or loss of buoyancy and in root length and appearance, should be noted. Significant features of the test medium (*e.g.* presence of undissolved material, growth of algae, fungi and bacteria in the test vessel) should also be noted.

37. In addition to determinations of main shoot length during the test, effects of the test chemical on three (or more) of the following measurement variables should be also assessed:

i. Total lateral branches length
ii. Total shoot length
iii. Total root length
iv. Fresh weight
v. Dry weight
vi. Number of whorls

Note 1: The observations made during the range-finding test could help in selecting relevant additional measurements among the six variables listed above.

Note 2: The determination of the fresh and dry weights (parameters iv and v) is highly desirable.

Note 3: Due to the fact that sucrose and light (exposure of roots to light during the test) may have an influence on auxin (plant growth hormone) transport carriers, and that some chemicals may have an auxin-type mode of action, the inclusion of root endpoints (parameter iii) is questionable.

Note 4: The ring test results show high coefficients of variation (> 60%) for the total lateral branch length (parameter i). Total lateral branch length is in any case encompassed within the total shoot length measurement (parameter ii) which shows more acceptable coefficients of variation of < 30%.

Note 5: Resulting from the above considerations, the recommended main measurement endpoints are: total shoot length, fresh weight and dry weight (parameters ii, iv and v); parameter vi – number of whorls – is left to the experimenter's judgment.

38. Main shoot length and number of whorls have an advantage, in that they can be determined for each test and control vessel at the start, during, and at the end of the test by photography and image analysis, although a (sterile) ruler can also be used.

39. Total lateral branches length, total root length (as a sum of all lateral branches or roots) and total shoot length (as a sum of main shoot length and total lateral branches length) can be measured with a ruler at the end of exposure.

40. The fresh and/or dry weight should be determined at the start of the test from a sample of the pre-culture representative of what is used to begin the test, and at the end of the test with the plant material from each test and control vessel.

41. Total lateral branches length, total shoot length, total root length, fresh weight, dry weight and number of whorls may be determined as follows:

i. Total lateral branches length: The lateral branch length may be determined by measuring all lateral branches with a ruler at the end of exposure. The total lateral branches length is the sum of all lateral branches of each test and control vessel.

ii. Total shoot length: The main shoot length may be determined by image analysis or using a ruler. The total shoot length is the sum of the total lateral branches length and the main shoot length of each test and control vessel at the end of exposure.

iii. Total root length: The root length may be determined by measuring all roots with a ruler at the end of exposure. The total root length is the sum of all roots of each test and control vessel.

iv. Fresh weight: The fresh weight may be determined by weighing the test organisms at the end of exposure. All plant material of each test and control vessel will be rinsed with distilled water, dabbed dry with cellulose paper. After this preparation the fresh weight will be determined by weighing. The starting biomass (fresh weight) is determined on the basis of a sample of test organisms taken from the same batch used to inoculate the test vessels.

v. Dry weight: After the preparations for the determination of the fresh weight the test organisms will be dried at 60 °C to a constant weight. This mass is the dry weight. The starting biomass

(dry weight) is determined on the basis of a sample of test organisms taken from the same batch used to inoculate the test vessels.

vi. Number of whorls: All whorls will be counted out along the main shoot.

Frequency of measurement and analytical determinations

42. If a static test design is used, the pH of each treatment should be measured at the beginning and at the end of the test. If a semi-static test design is used, the pH should be measured in each batch of 'fresh' test solution prior to each renewal and also in the corresponding 'spent' solutions.

43. Light intensity should be measured in the growth chamber, incubator or room at points in the same distance from the light source as from the test organisms. Measurements should be made at least once during the test. The temperature of the medium in a surrogate vessel held under the same conditions in the growth chamber, incubator or room should be recorded at least daily (or continuously with a data logger).

44. During the test, the concentrations of the test substance(s) are determined at appropriate intervals. In static tests, the minimum requirement is to determine the concentrations at the beginning and at the end of the test.

45. In semi-static tests where the concentrations of the test substance(s) are not expected to remain within ± 20% of the nominal concentration, it is necessary to analyse all freshly prepared test solutions and the same solutions at each renewal. However, for those tests where the measured initial concentrations of the test substance(s) are not within ± 20% of nominal but where sufficient evidence can be provided to show that the initial concentrations are repeatable and stable (*i.e.* within the range 80 – 120% of the initial concentration), chemical determinations may be carried out on only the highest and lowest test concentrations. In all cases, determination of test concentrations prior to renewal need only be performed on one replicate vessel at each test concentration (or the contents of the vessels pooled by replicate).

46. If there is evidence that the test concentration 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 ± 20 %, analysis of the results should be based on the geometric mean concentration during exposure or models describing the decline of the concentration of the test chemical (5).

Limit test

47. Under some circumstances, e.g. when a preliminary test indicates that the test chemical has no toxic effects at concentrations up to 100 mg/L or in case of a substance up to its limit of solubility in the test medium or in case of a formulation up to its limit of dispersibility, 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 is 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 doubled. Growth in the control and treatment group may be analysed using a statistical test to compare means, *e.g.* a Student's t-test.

DATA AND REPORTING

Response variables

48. The purpose of the test is to determine the effects of a test chemical on the vegetative growth of *Myriophyllum spicatum*. This Test Guideline describes two response variables.

a) Average specific growth rate: This response variable is calculated on the basis of changes in the logarithms of main shoot length, and in addition, on the basis of changes in the logarithms of other measurement parameters, *i.e.* total shoot length, fresh weight, dry weight or number of whorls over time (expressed per day) in the controls and each treatment group. Note: For the measurement parameter total lateral branches length and total root length a calculation of the average specific growth rate is not possible. At the beginning of the test, the test organism has no lateral branches and no roots (based on the preparation from the pre-culture); starting from the value zero, the calculation of the average specific growth rate is not defined.

b) Yield: This response variable is calculated on the basis of changes in main shoot length, and in addition, on the basis of changes in other measurement parameters – *i.e.* preferably total shoot length, fresh weight, dry weight or number of whorls, and other parameters if deemed useful – in the controls and in each treatment group until the end of the test.

49. Toxicity estimates should be based on main shoot length and three additional measurement variables (*i.e.* preferably total shoot length fresh weight, dry weight or number of whorls, see paragraph 37 and Notes 2, 4 and 5 to this paragraph), because some chemicals may affect other measurement variables much more than the main shoot length. This effect would not be detected by calculating main shoot length only.

Average specific growth rate

50. The average specific growth rate for a specific period is calculated as the logarithmic increase in the growth variables – main shoot length and three additional measurement variables (*i.e.* total shoot length, fresh weight, dry weight or number of whorls) – using the formula below for each replicate of control and treatments:

$$\mu_{i-j} = \frac{\ln(N_j) - \ln(N_i)}{t}$$

where:

- μ_{i-j} : average specific growth rate from time i to j
- N_i : measurement variable in the test or control vessel at time i
- N_j : measurement variable in the test or control vessel at time j
- t : time period from i to j

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

51. The average specific growth rate should be calculated for the entire test period (time "i" in the above formula is the beginning of the test and time "j" is the end of the test). For each test concentration and control, calculate a mean value for average specific growth rate along with the variance estimates. In

addition, the section-by-section growth rate should be assessed in order to evaluate effects of the test chemical occurring during the exposure period (*e.g.* by inspecting log-transformed growth curves).

52. Percent inhibition of growth rate (I_r) may then be calculated for each test concentration (treatment group) according to the following formula:

$$\% I_r = \frac{(\mu_C - \mu_T)}{\mu_C} \times 100$$

where:

- $\% I_r$: percent inhibition in average specific growth rate
- μ_C : mean value for μ in the control
- μ_T : mean value for μ in the treatment group

Yield

53. Effects on yield are determined on the basis of the measurement variable main shoot length and three additional measurement variables (*i.e.* preferably total shoot length, fresh weight, dry weight or number of whorls) present in each test vessel at the start and at the end of the test. For fresh weight or dry weight, the starting biomass is determined on the basis of a sample of test organisms taken from the same batch used to inoculate the test vessels. For each test concentration and control, calculate a mean value for yield along with variance estimates. The mean percent inhibition in yield ($\% I_y$) may be calculated for each treatment group as follows:

$$\% I_y = \frac{(b_c - b_T)}{b_c} \times 100$$

where:

- $\% I_y$: percent reduction in yield
- b_C : final biomass minus starting biomass for the control group
- b_T : final biomass minus starting biomass in the treatment group

Doubling time

54. To determine the doubling time (T_d) of main shoot length and adherence to this validity criterion (see paragraph 8), the following formula is used with data obtained from the control vessels:

$T_d = \ln 2/\mu$

Where μ is the average specific growth rate determined as described in paragraphs 50-52.

Plotting concentration-response curves

55. Concentration-response curves relating mean percentage inhibition of the response variable (I_r, or I_y calculated as shown in paragraph 53) and the log concentration of the test chemical should be plotted.

EC_x estimation

56. Estimates of the EC_x should be based upon both average specific growth rate (E_rC_x) and yield (E_yC_x), each of which should in turn be based upon main shoot length, and possibly additional measurement variables (*i.e.* preferably total shoot length, fresh weight, dry weight or number of whorls). This is because there are chemicals that impact main shoot length and other measurement variables differently. The desired toxicity parameters are therefore four EC_x values for each inhibition level x calculated: E_rC_x (main shoot length); E_rC_x (*i.e.* preferably total shoot length, fresh weight, dry weight, or number of whorls); E_yC_x (main shoot length); and E_yC_x (*i.e.* preferably total shoot length, fresh weight, dry weight or number of whorls).

57. It should be noted that EC_x values calculated using these two response variables are not comparable and this difference is recognised when using the results of the test. EC_x values based upon average specific growth rate (E_rC_x) will in most cases be higher than results based upon yield (E_yC_x) – if the test conditions of this Guideline are adhered to – due to the mathematical basis of the respective approaches. This difference should not be interpreted as a difference in sensitivity between the two response variables, simply the values are different mathematically.

Statistical procedures

58. 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 (7), 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 (7). 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 should be modified to accommodate growth rate or yield data. Specific procedures for determination of EC_x values from continuous data can be found in (8) (9) (10).

59. 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.

60. EC_{50} estimates and confidence limits may also be obtained using linear interpolation with bootstrapping (10), if available regression models/methods are unsuitable for the data.

61. For estimation of the LOEC and hence the NOEC, it is necessary to compare treatment means using analysis of variance (ANOVA) techniques. The mean for each concentration is then compared with the control mean using an appropriate multiple comparison or trend test method. Dunnett's or Williams'test may be useful (12) (13) (14) (15) (16). 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 (15). 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 (10).

62. 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 *Myriophyllum* test. However, 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} and their confidence limits should be reported.

Reporting

63. The test report includes the following:

Test chemical

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate).

Multi-constituent substance, UVBCs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Test species

- Scientific name and source.

Test conditions

- Test procedure used (static or semi-static).
- Date of start of the test and its duration.
- Test medium.
- Description of the experimental design: test vessels and covers, solution volumes, main shoot length per test vessel at the beginning of the test.
- Test concentrations (nominal and measured as appropriate) and number of replicates per concentration.
- Methods of preparation of stock and test solutions including the use of any solvents or dispersants.
- Temperature during the test.
- Light source, light intensity and homogeneity.
- pH values of the test and control media.
- The method of analysis of test chemical with appropriate quality assessment data (validation studies, standard deviations or confidence limits of analyses).

- Methods for determination of main shoot length and other measurement variables, *e.g.* total lateral branches length, total shoot length, total root length, fresh weight, dry weight or number of whorls.

- State of the culture (sterile or non-sterile) of each test and control vessel at each observation.

- All deviations from this Test Guideline.

Results

- Raw data: main shoot length and other measurement variables in each test and control vessel at each observation and occasion of analysis.

- Means and standard deviations for each measurement variable.

- Growth curves for each measurement variable.

- Calculated response variables for each treatment replicate, with mean values and coefficient of variation for replicates.

- Graphical representation of the concentration/effect relationship.

- Estimates of toxic endpoints for response variables e.g. EC_{50}, EC_{10}, EC_{20}, and associated confidence intervals. If calculated, LOEC and/or 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 visual signs of phytotoxicity as well as observations of test solutions.

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

LITERATURE

(1) ASTM Designation E 1913-04, Standard Guide for Conducting Static, Axenic, 14-Day Phytotoxicity Tests in Test Tubes with the Submersed Aquatic Macrophyte, *Myriophyllum sibiricum* Komarov.

(2) Maletzki, D. et al. (2010), *Myriophyllum spicatum* als ökotoxikologischer Testorganismus: Methodenentwicklung eines sedimentfreien Testsystems und erste Ergebnisse mit 3,5-Dichlorphenol, *Umweltwiss Schadst Forsch,* No. 22, pp. 702–710.

(3) OECD (2006), *Test No. 221: Lemna sp. Growth Inhibition Test*, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris. http://dx.doi.org/10.1787/9789264016194-en.

(4) OECD (2014), "Myriophyllum spicatum Toxicity Test: Results of an inter-laboratory ring test using a sediment-free test system", OECD Environment, Health and Safety Publications (EHS), Series Testing and Assessment, No. 205, OECD Publishing, Paris.

(5) OECD (2000), "Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures", OECD Environment, Health and Safety Publications (EHS), Series on Testing and Assessment, No. 23, OECD Publishing, Paris.

(6) OECD (2014) *Test No. 239: Water-Sediment Myriophyllum spicatum Toxicity Test*, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

(7) Christensen, E.R., N. Nyholm (1984), Ecotoxicological Assays with Algae: Weibull Dose-Response Curves, *Environmental Science & Technology*, Vol. 18/9, 713-718.

(8) Nyholm, N. et al. (1992), Statistical treatment of data from microbial toxicity tests, *Environmental Toxicology and Chem*istry, Vol. 11/2, pp. 157-167.

(9) Bruce, R.D., D.J. Versteeg (1992), A statistical procedure for modelling continuous toxicity data, *Environmental Toxicology and Chemistry*, Vol. 11/10, pp. 1485-1494.

(10) OECD (2006), "Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application", OECD Environment, Health and Safety Publications (EHS), Series on Testing and Assessment, No. 54, OECD Publishing, Paris.

(11) Norberg-King, T.J. (1988), An interpolation estimate for chronic toxicity: The ICp approach, National Effluent Toxicity Assessment Center Technical Report 05-88, US EPA, Duluth, MN.

(12) Dunnett, C.W. (1955), A multiple comparisons procedure for comparing several treatments with a control, *Journal of the American Statistical Association*, Vol. 50/272, pp. 1096-1121.

(13) Dunnett, C.W. (1964), New tables for multiple comparisons with a control, *Biometrics*, Vol. 20/3, pp. 482-491.

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

(15) Williams, D.A. (1972), The comparison of several dose levels with a zero dose control, *Biometrics*, Vol. 28/2, pp. 519-531.

(16) Brain, P., R. Cousens (1989), An equation to describe dose-responses where there is stimulation of growth at low doses, *Weed Research*, Vol. 29/2, pp. 93-96.

ANNEX 1

DEFINITIONS

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

Biomass is the fresh and/or dry weight of living matter present in a population. In this test the biomass is the sum of main shoot, all lateral branches and all roots.

Chlorosis is the change of the color from green to yellowing of test organism especially of the whorls.

EC_x is the concentration of the test chemical dissolved in test medium that results in a x% (*e.g.* 50%) reduction in growth of *Myriophyllum spicatum* 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, followed by the measurement variable used, *e.g.* E_rC (main shoot length).

Growth is an increase in the measurement variable, *e.g.* main shoot length, total lateral branches length, total shoot length, total root length, fresh weight, dry weight or number of whorls, over the test period.

Growth rate (average specific growth rate) is the logarithmic increase in the measurement variable during the exposure period. *Note*: Growth rate related response variables are independent of the duration of the test as long as the growth pattern of unexposed control organisms is exponential.

Lowest Observed Effect Concentration (LOEC) is the lowest tested concentration at which the chemical 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 should have a harmful effect equal to or greater than those observed at the LOEC. When these two conditions cannot be satisfied, a full explanation should be given for how the LOEC (and hence the NOEC) has been selected.

Measurement variables are any type of variables which are measured to express the test endpoint using one or more different response variables. In this guideline main shoot length, total lateral branches length; total shoot length, total root length, fresh weight, dry weight and number of whorls are measurement variables.

Monoculture is a culture with one plant species.

Necrosis is dead (i.e. white or dark brown) tissue of the test organism.

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 variable describing biomass by different methods of calculation. For this guideline growth rate and yield are response variables derived from measurement variables like main shoot length, total shoot length, fresh weight, dry weight, or number of whorls.

Semi-static (renewal) test is a test in which the test solution is periodically replaced at specific intervals during the test.

Static test is a test method without renewal of the test solution during the test.

Test endpoint describes the general factor that will be changed relative to control by the test chemical as aim of the test. In this guideline the test endpoint is inhibition of growth which may be expressed by different response variables which are based on one or more measurement variables.

Test medium is the complete synthetic growth medium on which test plants grow when exposed to the test chemical. The test chemical will normally be dissolved in the test medium.

Yield is value of a measurement variable to express biomass at the end of the exposure period minus the measurement variable at the start of the exposure period. Note: When the growth pattern of unexposed organisms is exponential, yield-based response variables will decrease with the test duration.

ANNEX 2

MODIFIED ANDREWS' MEDIUM FOR STOCK CULTURE AND PRE-CULTURE

From five separately prepared nutrient stock solutions the modified Andrews' medium required for stock culture and pre culture will be prepared, with addition of 3 % sucrose.

Table 1: Composition of Andrews' nutrient solution: (ASTM Designation E 1913-04)

Production of nutrient stock solutions			**Production of nutrient solution**
Stock solution	**Chemical**	**Initial weight per 1000 ml**	**ml per 5 L nutrient solution**
1	KCl	74,6 mg	50
	KNO_3	8,08 g	
	$Ca(NO_3)_2 * 4 H_2O$	18,88 g	
2	$MgSO_4 *7 H_2O$	9,86 g	50
3	See below stock solution 3.1		50
4	KH_2PO_4	2,72 g	50
5	$FeSO_4 * 7 H_2O$	0,278 g	50
	$Na_2EDTA* 2 H_2O$	0,372 g	

Stock solutions can be kept in a refrigerator for 6 months (at 5-10 °C). Only stock solution No. 5 has a reduced shelf life (two months).

Table 2: Production of **stock solution 3.1** for preparing stock solution 3

Chemical	**Initial weight g/100 ml**
$MnSO_4 * 4 H_2O$	0,223
$ZnSO_4 * 7 H_2O$	0,115
H_3BO_3	0,155
$CuSO_4 * 5 H_2O$	0,0125
$(NH_4)_6Mo_7O_{24} * 4 H_2O$	0,0037

After having produced stock solution 3.1 (Table 2), deep-freeze this solution in approximately 11 ml-aliquots (at -18°C at least). The deep-frozen portions have a shelf life of five years.

To produce stock solution 3, defrost stock solution 3.1, fill 10 ml of it into a 1 L volumetric flask and add ultra-pure water up to the flask‘s mark.

To obtain modified Andrews' medium, fill approximately 2500 ml ultra-pure water into a 5 L volumetric flask. After adding 50 ml of each stock solution, fill 90% of the volumetric flask with ultra-pure water and set pH to 5.8.

After this, add 150 g dissolved sucrose (3% per 5 L); then, fill the volumetric flask with ultra-pure water up to the mark. Finally, the nutrient solution is filled into 1 L Schott flasks and autoclaved at 121 °C for 20 minutes.

The nutrient solution thus yielded can be kept sterile in a refrigerator (at 5-10 °C) for three months.

MODIFIED ANDREWS' MEDIUM FOR SEDIMENT-FREE TOXICITY TEST

From the five nutrient stock solutions already mentioned in Tables 1 and 2, a tenfold concentrated, modified Andrews' medium required for obtaining the test solutions will be prepared, with addition of 30% sucrose. To do so, fill approximately 100 ml ultra-pure water into a 1 L volumetric flask. After adding 100 ml of each of the stock solutions, set pH to 5.8. After this, add 30% dissolved sucrose (300 g per 1000 ml); then, fill the volumetric flask with ultra-pure water up to the mark.

Finally, the nutrient solution is filled into 0.5 L Schott flasks and autoclaved at 121 °C for 20 minutes.

The tenfold concentrated modified nutrient solution thus yielded can be kept sterile in a refrigerator (at 5-10 °C) for three months.

ANNEX 3

MAINTENANCE OF STOCK CULTURE

In this Annex 3 the stock culture of *Myriophyllum spicatum* L[1], a submersed aquatic dicotyledon, a species of the water milfoils family is described. Between June and August, inconspicuous pink-white flowers protrude above the water surface. The plants are rooted in the ground by a system of robust rhizomes and can be found in the entire northern hemisphere in eutrophic, however non-polluted and more calciferous still waters with muddy substrate. *Myriophyllum spicatum* prefers fresh water, but is found in brackish water as well.

For sediment-free stock culture under laboratory conditions, sterile plants are required. Sterile plants are available from the ecotoxicology laboratory of the German Umweltbundesamt (Federal Environment Agency of Germany).

Alternatively, test organisms can be prepared from non-sterile plants in accordance with ASTM designation E 1913-04. See below – extracted from the ASTM Standard Guide – the procedure for culturing *Myriophyllum sibiricum* collected from field:

"If starting from field collected, non-sterile plants, collect *M. sibiricum* turions in the autumn. Place the turions into a 20-L aquarium containing 5 cm of sterile sediment that is covered with silica sand or Turface® and 18 L of reagent water. Aerate the aquarium and maintain at a temperature of 15 °C and a fluence rate of 200 to 300 µmol m^{-2} s^{-1} for 16 h per day. The plant culture in the aquarium may be maintained as a backup source of plants in case the sterile plant cultures are destroyed by mechanical malfunction in the growth cabinet, contamination, or other reason. The plants grown in the aquarium are not sterile and sterile cultures cannot be maintained in a batch culturing system. To sterilize the culture, plants are removed from the aquarium and rinsed under flowing deionized water for about 0.5 h. Under aseptic conditions in a laminar airflow cabinet, the plants are disinfected for less than 20 min (until most of the plant tissue is bleached and just the growing apex is still green) in a 3% (w/v) sodium hypochlorite solution containing 0.01 % of a suitable surfactant. Agitate the disinfectant and plant material. Segments with several nodes are transferred into sterile culture tubes containing 45 mL of sterilized modified Andrews' medium and capped with plain culture tube closures. Only one plant segment is placed into each test chamber. Laboratory sealant film is used to secure the closure to the culture vessel. Once a sterile culture has been established, plant segments containing several nodes should be transferred to new test chambers containing fresh liquid nutrient media every ten to twelve days. As demonstrated by culturing on agar plates, the plants must be sterile and remain sterile for eight weeks before testing can be initiated."

Since the modified Andrews' medium contains sucrose (which stimulates the growth of fungi and bacteria), all material, solutions and culturing be conducted under sterile conditions. All liquids as well as equipment are sterilized before use. Sterilization is carried out via heated air treatment (210 °C) for 4 hours or autoclaving for 20 minutes at 121 °C. In addition, all flasks, dishes, bowls etc and other equipment undergo flame treatment at the sterile workbench just prior to use.

[1] Carl von Linné (* May, 23th, 1707 in Råshult /Älmhult; † January, 10th, 1778 in Uppsala).

Stock cultures can be maintained under reduced illumination and temperature (50 µE m^{-2} s^{-1}, 20 ± 2 °C) for longer times without needing to be re-established. The *Myriophyllum* growth medium should be the same as that used for testing but other nutrient rich media can be used for stock cultures.

The plant segments are distributed axenically over several 500 ml Erlenmeyer or/and 2000 ml Fernbach flasks, each filled with approximately 450 respectively 1000 ml modified Andrews' medium. Then, the flasks are axenically cellulose plug stoppered.

In addition, thorough flame treatment of equipment at the sterile workbench just prior to use is absolutely necessary. Dependent on number and size, the plants are to be transferred into fresh nutrient solution approximately every three weeks.

Apices as well as segments of the stem middle part for this renewed culture can be used. Number and size of transferred plants (or segments of plants) are dependent on how many plants are needed. For example, you can transfer five shoot segments into one Fernbach flask and three shoot segments into one Erlenmeyer flask, each with a length of 5 cm. Discard any rooted, flowering, dead or otherwise conspicuous parts.

Figure 1: Cutting of plants for the stock and pre culture after 3 weeks of cultivation.

Culturing of plants is to be performed in 500 ml Erlenmeyer and 2000 ml Fernbach flasks in a cooling incubator at 20 ± 2 °C with continuously light at approximately 100-150 µE m^{-2} s^{-1} or 6000-9000 Lux (emitted by chamber illumination with colour temperature "warm white light").

Figure 2: Culturing of plants in a cooling incubator with chamber illumination.

Chemically clean (acid-washed) and sterile glass culture vessels should be used and aseptic handling techniques employed. In the event of contamination of the stock culture *e.g.* by algae, fungi and/or bacteria a new culture should be prepared or a stock culture from another laboratory should be used to renewal of the one culture.

ANNEX 4

MAINTENANCE OF PRE-CULTURE AND PREPARATION OF TEST ORGANISM FOR TESTING

To obtain pre-culture, cut shoots of stock culture into segments with two whorls each; put segments into Fernbach flasks filled with modified Andrews' medium (with 3% sucrose). Each flask can contain up to 50 shoot segments. However, care is to be taken that the segments are vital and do not have any roots and lateral branches or their buds (see figure 1 in Annex 3).

The pre-culture organisms are cultured for 14 to 21 days under sterile conditions in an environmental chamber with alternating 16/8 hour light/dark phases. Light intensity selected from the range of 100-150 $\mu E\ m^{-2}\ s^{-1}$. The temperature in the test vessels should be 23 ± 2 °C.

Since the modified Andrews' medium contains sucrose (which stimulates the growth of algae, fungi and bacteria), test chemical solutions should be prepared and culturing be conducted under sterile conditions. All liquids as well as equipment are sterilized before use. Sterilization is carried out via heated air treatment (210 °C) for 4 hours or autoclaving for 20 minutes at 121 °C. In addition, all flasks, dishes, bowls etc. and other equipment undergo flame treatment at the sterile workbench just prior to use.

Shoots are axenically removed from the pre-culture flasks, choosing material that is as homogeneous as possible. Each testing requires at least 60 test organisms (testing with eight test chemical concentrations). For testing, take fresh lateral branches from pre-cultures, shorten them to 2.5 cm from base (measured with ruler) and transfer them into a beaker containing sterile modified Andrews' medium. These fresh lateral branches can be used for the sediment-free *Myriophyllum spicatum* toxicity test.

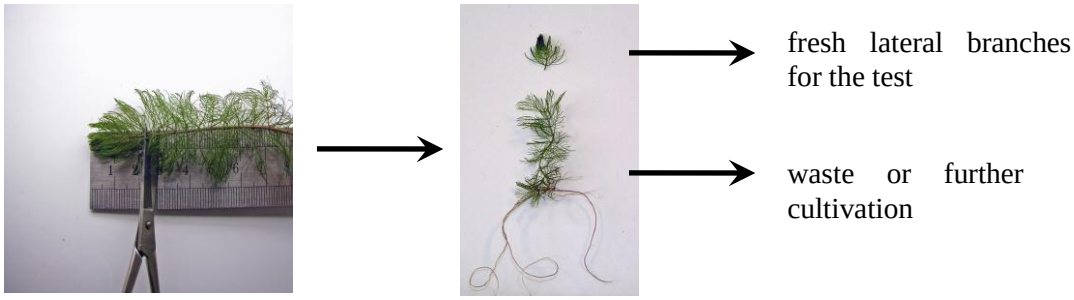

Figure 2: Cutting of plants from the pre culture for the sediment-free *Myriophyllum spicatum* toxicity test.