Section 2
Effects on Biotic Systems

Test Guideline No. 201

Freshwater Alga and Cyanobacteria, Growth Inhibition Test



2 July 2026

OECD Guidelines for the Testing of Chemicals

OECD
BETTER POLICIES FOR BETTER LIVES

This work was approved and declassified by the Chemicals and Biotechnology Committee on 15/06/2026.

Please cite this publication as:

OECD (2026), *Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test,* OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264069923-en.

© OECD 2026

Attribution 4.0 International (CC BY 4.0)

This work is made available under the Creative Commons Attribution 4.0 International licence. By using this work, you accept to be bound by the terms of this licence (https://creativecommons.org/licenses/by/4.0/).

Attribution – you must cite the work.

Translations – you must cite the original work, identify changes to the original and add the following text: *In the event of any discrepancy between the original work and the translation, only the text of original work should be considered valid.*

Adaptations – you must cite the original work and add the following text: *This is an adaptation of an original work by the OECD. The opinions expressed and arguments employed in this adaptation should not be reported as representing the official views of the OECD or of its Member countries.*

Third-party material – the licence does not apply to third-party material in the work. If using such material, you are responsible for obtaining permission from the third party and for any claims of infringement.

You must not use the OECD logo, visual identity or cover image without express permission or suggest the OECD endorses your use of the work.

Any dispute arising under this licence shall be settled by arbitration in accordance with the Permanent Court of Arbitration (PCA) Arbitration Rules 2012. The seat of arbitration shall be Paris (France). The number of arbitrators shall be one.

About the OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 38 countries in North and South America, Europe and the Asia and Pacific region, as well as the European Union, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialised committees and working groups composed of member country delegates. Observers from several Partner countries and from interested international organisations attend many of the OECD's workshops and other meetings. Committees and working groups are served by the OECD Secretariat, located in Paris, France, which is organised into directorates and divisions.

The Environment, Health and Safety Division publishes free-of-charge documents in twelve different series: **Testing and Assessment**; **Good Laboratory Practice and Compliance Monitoring**; **Pesticides**; **Biocides**; **Risk Management**; **Harmonisation of Regulatory Oversight in Biotechnology**; **Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents; Safety of Manufactured Nanomaterials;** and **Adverse Outcome Pathways.** More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (https://www.oecd.org/en/topics/chemical-safety-and-biosafety.html).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organizations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank, Basel, Rotterdam and Stockholm Conventions and OECD. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

© OECD (2026)

OECD/OCDE 201

Adopted:
2 July 2026

OECD GUIDELINES FOR THE TESTING OF CHEMICALS

Freshwater Alga and Cyanobacteria, Growth Inhibition Test

© OECD (2026)

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 (originally 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 leading to the publication of a revised version in 2006 and a further correction in 2011. 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. This latest update includes:

- the diatom strain *Mayamaea permitis* as new recommended species (21)
- an update of scientific name for the recommended strains (*Pseudokirchneriella subcapitata*, *Navicula pelliculosa*, and *Anabaena flos-aquae*),
- a clarification on the calculation of coefficient of variation (CV) of the daily/section-by-section specific growth rates.

2. Definitions used are given in Annex 1.

3. The purpose of this test is to determine the effects of a chemical on the growth of freshwater microalgae and/or cyanobacteria. Exponentially growing test organisms are exposed to the test chemical 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 chemical. 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 and other measures for which the conversion factor between the measured surrogate parameter and biomass is known. 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_rC_x (e.g. E_rC_{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_yC_{50}).

© OECD (2026)

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

9. When this Test Guideline is used for the testing of a mixture for a regulatory purpose, consideration should be given to whether the Test Guideline is appropriate for that purpose and, if so, to the rationale for expecting it to provide adequate results. Such consideration is not required when the testing of the mixture is expressly mandated by regulation. Mixture is defined as a deliberate physical mixture of test chemicals that does not produce a substance different from the substances that were combined. The details can be found in Guidance Document 23 (2).

Information on the test chemical

10. Information on the test chemical 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, acid dissociation constant (pKa), and results of studies of transformation including biodegradability in water.

11. The water solubility, octanol water partition coefficient (P_{ow}) and vapour pressure of the test chemical 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

12. 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 daily/section-by-section specific growth rates (days 0-1, 1-2 and 2-3, for 72-hour tests) in the control cultures should not exceed 35% (See Annex 1 for the definition of “coefficient of variation” and Annex 6 for an example calculation of a CV for daily/section-by-section specific growth rates).
- The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures should not exceed 7% in tests with *Raphidocelis subcapitata* and *Desmodesmus subspicatus*. For other less frequently tested species, the value should not exceed 10%.

Reference substance

13. Reference substance(s), such as 3,5-dichlorophenol and potassium dichromate used in international ring tests (1), may be tested as a means of checking the test procedure. The examples of test concentration and EC50 variability are provided in (1). It is desirable to test a reference substance at least twice a year.

© OECD (2026)

Applicability of the test

14. This Guideline is most easily applied to water-soluble chemicals which, under the conditions of the test, are likely to remain in the water. For testing of chemicals that are volatile, strongly adsorbing, coloured, having a low solubility in water or chemicals 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). For testing nanomaterials, refer to the specific guide (5).

Description of the method

Apparatus

15. 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.

16. 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 CO_2 from the atmosphere (see paragraph 30). Note that the liquid volume should be sufficient for analytical determinations (see paragraph 37).

17. 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 ± 2°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 (4 π) receptor (which responds to direct and reflected light from all angles above and below the plane of measurement), or a 2 π 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 performed using an electronic particle counter, a microscope with counting chamber, or a flow cytometer. Other biomass surrogates can be measured using a 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

18. 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.

19. 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. In the cases where the prescribed criteria (specifically the section-by-section specific growth rates validity criterion) are not met, consideration of broader test factors, such as cell culturing and cell counting methods, may be needed.

© OECD (2026)

Growth medium

20. 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.

21. 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 (2) (4).

Initial biomass concentration

22. The initial biomass in the test cultures should be the same in all test cultures and sufficiently low to allow exponential growth throughout the incubation period without risk of nutrient depletion. The following initial cell concentrations are recommended:

Raphidocelis subcapitata:	$5 \times 10^3 - 10^4$	cells/mL
Desmodesmus subspicatus	$2\text{-}5 \times 10^3$	cells/mL
Mayamaea permitis (formerly *Navicula pelliculosa*)	$5 \times 10^3 - 10^4$	cells/mL
Anabaena variabilis	10^4	cells/mL
Synechococcus leopoliensis	$5 \times 10^4 - 10^5$	cells/mL

Concentrations of test chemical

23. 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 chemicals 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

24. The test design should include three replicates at each test concentration. If determination of the NOEC is not required, then the test design may be altered to increase the number of concentrations for obtaining robust E_rC_x, whereas the number of replicates per concentration may be reduced. The number of control replicates should be at least three, and ideally should be twice the number of replicates used for each test concentration.

25. A separate set of test solutions may be prepared for analytical determinations of test chemical concentrations (see paragraphs 37 and 39).

26. When a solvent is used to solubilise the test chemical, additional controls containing the solvent at the same concentration as used in the test cultures should be included in the test design.

© OECD (2026)

Preparation of inoculum culture

27. 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

28. All test solutions should 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 chemical with growth medium and inoculum culture. Inoculum should be impartially or randomly distributed among the test vessels in such a manner that test results show no significant bias from the distributions, by using randomly generated integer numbers with no duplicates. Stock solutions are normally prepared by dissolving the chemical in test medium. It is advisable to limit the presence of undissolved test chemical.

29. 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: solvent control) in the test series. In this case, a solvent control is mandatory in addition to a control without solvent.

Incubation

30. 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 CO_2. 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 to minimise differences in light intensity between flasks (see paragraph 32 for light condition),e.g., by using randomly generated integer numbers with no duplicates.

31. 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 CO_2 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 CO_2 by reducing the initial biomass or the test duration.

32. 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 $\mu E \cdot m^{-2}\ s^{-1}$ when measured in the photosynthetically effective wavelength range of 400-700 nm using an appropriate receptor. Some species, in particular *Anabaena variabilis*, 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 $\mu E \cdot m^{-2} \cdot s^{-1}$ should be selected. (For light-measuring

© OECD (2026)

instruments calibrated in lux, an equivalent range of 4440 – 8880 lux for cool white light corresponds approximately to the recommended light intensity 60-120 $\mu E \cdot m^{-2} \cdot s^{-1}$). Maintain the light intensity within ±15% from the average light intensity over the incubation area.

Test duration

33. Test duration is normally 72 hours. However, shorter or longer test durations may be used provided that all validity criteria in paragraph 12 can be met.

Measurements and analytical determinations

34. 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.

35. Measurement of biomass is done by manual cell counting by microscope with counting chamber or an electronic particle counter (by cell counts and/or biovolume). Alternative techniques, e.g. flow cytometry, *in vitro* or *in vivo* chlorophyll fluorescence (6) (7), or optical density can be used if a satisfactory correlation with biomass can be demonstrated over the range of biomass occurring in the test.

36. Measure the pH of the solutions at the beginning and at the end of the test. The pH at the beginning of the test can be measured in a sample of the test solutions not used for the test, prior to the addition of the algal inoculum.

37. Provided an analytical procedure for determination of the test chemical 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.

38. Analysis of the concentration of the test chemical, 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 chemicals, additional samplings for analysis at 24 hour intervals during the exposure period are recommended in order to better define loss of the test chemicals. For these chemicals, extra replicates may be needed. In all cases, determination of test chemical concentrations need only be performed on one replicate vessel at each test concentration (or the contents of the vessels pooled by replicate).

39. 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 chemical 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. In this case, chemicals on the surface of, or inside, the algae will not be measured.

40. If there is evidence that the concentration of the chemical 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 chemical (8).

© OECD (2026)

41. 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 chemical 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 chemical 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 chemical (8) 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

42. 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 chemical) at the end of the test.

Limit test

43. 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 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

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

45. 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.

46. 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 clearly in the test report reasons for rejection of a data point as an outlier. Accepted reasons are only (rare) procedural mistakes and

© OECD (2026)

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

47. The purpose of the test is to determine the effects of the test chemical 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.

48. It should be noted that toxicity values calculated by using these two response variables are not comparable and this difference should be mentioned when using the results of the test. EC_x values based upon average specific growth rate (E_rC_x) will generally 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 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 or on test duration. In contrast, results based upon the yield response variable are dependent upon both variables. E_yC_x 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

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

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

Where:

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

X_i is the biomass at time i (days)

X_j is the biomass at time j (days)

For each treatment group and control group, calculate an average specific growth rate with variance estimates.

© OECD (2026)

50. 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 daily/section-by-section specific growth rate using equation [1], calculated as specified in Annex 1 (see also an example of these calculations in Annex 6) and examine whether the control growth rate remains constant (see section "Validity of the Test", paragraph 12). 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 chemical (including sorption onto the algal biomass) after initial exposure. Hence the daily/section-by-section growth rate may be assessed in order to evaluate effects of the test chemical occurring during the exposure period. Substantial differences between the daily/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.

51. 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.

52. When solvents are used to prepare the test solutions, and two controls (with and without solvent) do not differ statistically, they may be pooled for further analysis, e.g. for the derivation of NOE_rC or the estimation of E_rC_x values. If there is a statistically significant difference between the control and the solvent control, the solvent control should be used for the calculation of percent inhibition, taking into account the discretion of the regulatory authority. For a statistical procedure, see paragraph 61.

Yield

53. 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.

© OECD (2026)

Statistical procedure for E_rC_x estimation

54. Plot the average specific growth rate, including that of control, against the logarithm of the test chemical concentration and fit a smooth line through the data points by regression models to get the concentration-response relationship, with a model-based regression.

55. 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 (9). 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 or biomass data. For example, the log-logistic model (derived from the logit model), the log-normal model (derived from the probit model), the two types of Weibull model, and the Hill model may be used. In cases where growth stimulation is observed (see paragraph 62 “growth stimulation),a non-monotone model (including hormesis effect) may be used. Specific procedures for the determination of EC_x values from continuous data are described in (10) (11) (12) and (13). The use of non-linear regression analysis is further detailed in Annex 5.

56. For each response variable to be analysed, use the concentration-response relationship to calculate point estimates of EC_x values. 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. Outlier removal should be strongly justified by a documented deviation in test parameters (e.g., different light intensity or a contamination in one replicate) and supported by an appropriate statistical test used to evaluate the suspected outliers. Outlier removal 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.

57. EC_{50} estimates and confidence limits may also be obtained using linear interpolation with bootstrapping (15), if available regression models/methods are unsuitable for the data.

58. Criteria such as Normalized Width (NW) or Certainty of Protection Level can help mode selection.

Statistical procedure for NOE_rC estimation

59. For estimation of the LOEC and hence the NOEC, for effects of the test chemical on growth rate, the statistical approach should be selected after checking the assumptions relevant for growth as a continuous variable. In general, a one-side test is appropriate for a growth inhibition test. These assumptions include homogeneity of variance, approximately normality of the residuals, and monotonicity. Assumption checks should be assessed by graphical residual diagnostics (19) and, where appropriate, formal tests such as Levene’s or Bartlett’s. Based on the outcome, the most powerful appropriate test should be applied. For example, if homogeneity of variance is assumed, Dunnett’s or Williams’ test may be selected, whereas Steel’s test may be appropriate in cases of heterogeneity of variance (14) (16) (17) (18) (19). Additional guidance on determining the NOEC can be found in (12). Note that monotonicity is an assumption about the underlying population response. This means that even if the observed sample means are not strictly monotone and a monotonic trend test is not significant, a monotone trend should still be assumed if the test chemical is expected to inhibit growth, provided there is no indication of hormesis (“toxic stimulation”).”

60. Recent scientific developments have led to a recommendation of abandoning the concept of NOEC and replacing it with regression-based point estimates EC_x. Although the use of the EC_{10} or EC_5 is widely accepted, an

© OECD (2026)

appropriate value for x has not been established for this algal test. A range of 5 to 10 % appears to be appropriate (depending on the response variable chosen), and preferably both the EC_5 and EC_{10} should be reported.

Two-sample comparison procedure

61. For two-sample comparisons corresponding—either between the control and the test concentration in a limit test, or between a solvent control and a control without solvent—the appropriate statistical test should be selected depending on whether normality and, subsequently, homogeneity of variance are verified.

Growth stimulation

62. Growth stimulation (negative inhibition) at low concentrations is sometimes observed. This can result from either hormesis or from addition of stimulating growth factors with the test chemical 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. If growth stimulation is observed, both a standard monotone concentration-response model and an appropriate hormesis model (e.g. Brain-Cousens) (20) should be fitted. The appropriate model should then be selected based on goodness-of-fit/diagnostic criteria (i.e., AIC, “residuals vs. fitted values” plot, lack-of-fit test and checking stat. significance of model parameters), and the model selection should be reported. It neither model provides an acceptable fit, a non-parametric fallback, such as linear interpolation with bootstrapping, should be used. Growth stimulation should be ignored only if the effect size of growth stimulation is less than 10% compared with the control and the difference is not statistically significant (i.e., based on an appropriate post-hoc test).

Non toxic growth inhibition

63. Light absorbing test chemicals 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).

Test report

64. The test report should include the following:

Test chemical:

- 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.
- Date and result of the latest test performed with reference substance (3,5 DCP or potassium dichromate)

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;

© OECD (2026)

- 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 chemical 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., EC_{50}, EC_{10}, EC_{20} 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;
- discussion of the results, including any influence on the outcome of the test resulting from deviations from this Guideline.

© OECD (2026)

Literature

1) OECD (2026). Validation report for the revised Test Guideline TG 201, inclusion of a new algal strain, *Mayamaea permitis* (NIES-2724) in the Freshwater Alga and Cyanobacteria, Growth Inhibition Test. Series on Testing and Assessment No. 431. OECD Publications, Paris. Available at: https://www.oecd.org/en/topics/sub-issues/testing-of-chemicals/publications-on-testing-and-assessment-of-chemicals.html

2) OECD (2019), *Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures*, OECD Series on Testing and Assessment, OECD Publishing, Paris, https://doi.org/10.1787/0ed2f88e-en.

3) International Organisation for Standardisation (2008). ISO/TR 14442: Water quality — Guidelines for algal growth inhibition tests with poorly soluble materials, volatile compounds, metals and waste water.

4) International Organisation for Standardisation (2017). ISO 5667-16 Water quality – Sampling – Part 16: Guidance on Biotesting of Samples.

5) OECD (2025), *Guidance Document on Aquatic and Sediment Toxicological Testing of Nanomaterials, Second Edition*, OECD Series on Testing and Assessment, No. 420, OECD Publishing, Paris, https://doi.org/10.1787/17b3ce6f-en.

6) Mayer, P., Cuhel, R. and Nyholm, N. (1997). A simple in vitro fluorescence method for biomass measurements in algal growth inhibition tests. *Water Research* 31: 2525-2531.

7) Slovacey, R.E. and Hanna, P.J. (1997). In vivo fluorescence determinations of phytoplancton chlorophyll, Limnology & Oceanography 22, 5, pp.919-925

8) Simpson, S.L., Roland, M.G.E., Stauber, J.L. and Batley, G.E. (2003) Effect of declining toxicant concentrations on algal bioassay endpoints. *Environ. Toxicol. Chem.* 22, 2073-2079.

9) Christensen, E.R., Nyholm, N. (1984). Ecotoxicological Assays with Algae: Weibull Dose- Response Curves. *Env. Sci. Technol*. 19, 713-718.

10) Nyholm, N. Sørensen, P.S., Kusk, K.O. and Christensen, E.R. (1992): Statistical treatment of data from microbial toxicity tests. *Environ. Toxicol. Chem*. 11, 157-167.

11) Bruce, R.D.,and Versteeg, D.J. (1992). A statistical procedure for modelling continuous toxicity data. *Environ. Toxicol. Chem*. 11:1485-1494.

12) OECD (2006), *Current Approaches in the Statistical Analysis of Ecotoxicity Data: A guidance to application (annexes to this publication exist as a separate document)*, OECD Series on Testing and Assessment, No. 54, OECD Publishing, Paris, https://doi.org/10.1787/9789264085275-en

13) Green, J.G., Springer, T.A. and Holbech H. (2018) Statistical Analysis of Ecotoxicity Studies. John Willey & Sons, Inc., NJ, USA

14) Dunnett, C.W. (1955) A multiple comparisons procedure for comparing several treatments with a control. J. Amer. Statist. Assoc. 50: 1096-1121.

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

© OECD (2026)

16) Dunnett, C.W. (1964) New tables for multiple comparisons with a control. Biometrics 20: 482- 491.

17) 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.

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

19) Draper, N.R. and Smith, H. (1981). Applied Regression Analysis, second edition. Wiley, New York.

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

© OECD (2026)

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 (CV) 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 CV of the daily/section-by-section specific growth rate in replicate control cultures should be calculated as follows:

Calculate the standard deviation (SD) of the daily/section-by-section specific growth rates over the entire test period for the respective replicate;

Calculate CV (%) by dividing the SD calculated under point 1 by the average specific growth rate for the respective replicate;

Calculate the mean value out of all values calculated under point 2 to get the mean CV (%) of the daily/section by section specific growth rate in replicate control cultures[1].

EC_x is the concentration of the test chemical 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 chemical. The test chemical will normally be dissolved in the test medium.

Growth rate (average specific growth rate) is the logarithmic increase in biomass during the exposure period, expressed per day. The average specific growth rate is identical to the mean value of the daily/section-by-section specific growth rates.

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 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.

[1] An example of calculation is presented in Annex 6

© OECD (2026)

Mixture is a deliberate physical mixture of test chemicals that do not produce a substance that is different from the substances that were combined, including a prepared formulation or hydrate. Mixtures or preparations are generally fully characterised in terms of their components

No Observed Effect Concentration (NOEC) is the test concentration immediately below the LOEC. The NOEC may also be determined using non-linear regression.

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

Strain is a particular isolate of that species, derived from a single original sample.

Test chemical includes items such as natural substances, micro-organisms, etc.

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.

© OECD (2026)

Annex 2. Strains shown to be suitable for the test

Green algae

- *Raphidocelis subcapitata*, (formerly known as *Selenastrum capricornutum* = *Pseudokirchneriella subcapitata*), ATCC 22662, CCAP 278/4, 61.81 SAG, NIES-35, CPCC 37
- *Desmodesmus subspicatus* (formerly known as *Scenedesmus subspicatus*) 86.81 SAG, NIES-4248

Diatoms

- *Mayamaea permitis*, e,g, strains NIES-2724 or SAG 1050-3 (selected as the species most closely related to the lost recommended strain UTEX 664)

  Notes: *Mayamaea* sp. UTEX 664 (This strain was formerly misidentified as *Navicula pelliculosa* (= *Fistulifera pelliculosa*), but is not commercially available).

  Navicula pelliculosa, SAG 1050-3 (This strain was recently renamed "*Mayamaea permitis*" and is commercially available at SAG: Collection of Algal Cultures, Göttingen, Germany).

Cyanobacteria

- *Anabaena variabilis* (= *Trichormus variabilis*), UTEX 1444, ATCC 29413, CCAP 1403/13A, NIES-2095, CPCC67 (These strains were formerly misidentified as *Anabaena flos-aquae*)
- *Synechococcus leopoliensis* (= *Romeria leopoliensis*), UTEX 625, CCAP 1405/1, NIES-3277, CPCC 102

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

SAMS, Limited

SAMS

© OECD (2026)

Dunbeg, OBAN
PA 37 1QA,
Scotland, UK

CPCC, Canadian Phycological Culture Centre
Department of Biology
University of Waterloo
200 University Avenue West
Waterloo, ON N2L 3G1
Canada

NIES Microbial Culture Collection
National Institute for Environmental Studies
Tsukuba, Ibaraki 305-8506
Japan

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.

© OECD (2026)

LITERATURE

Krienitz L, Bock C, Nozaki H, Wolf M (2011) SSU rRNA gene phylogeny of morphospecies affiliated to the bioassay alga "*Selenastrum capricornutum*" recovered the polyphyletic origin of crescent-shaped chlorophyta. J Phycol. 47:880-893.

Thiel, T. et al. (2014) Complete genome sequence of *Anabaena variabilis* ATCC 29413. Standards in Genomic Sciences 9: 562-573.

Tsuji A., Suzuki S., Yamagishi T., Yamaguchi H. (2021). Alternative diatom strains for growth inhibition tests (OECD Test Guideline 201). Bull. Natl. Mus. Nat. Sci. Ser. B. 47: 183-192.

Lange-Bertalot, H. (1997) *Frankophila*, *Mayamaea* und *Fistulifera*: drei neue Gattungen der Klasse Bacillariophyceae. Archiv für Protistenkunde 148: 65-76.

Appearance and characteristics of recommended species

	R. subcapitata	*D. subspicatus*	*M. permitis*	*A. variabilis*	*S. leopoliensis*
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 (μm^3/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	1.4-1.8	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 µE m^{-2} s^{-1} and 21 °C

Specific Recommendations on Culturing and Handling of Recommended Test Species

Raphidocelis 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 variabilis

© OECD (2026)

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 variabilis 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 should be identical for each treatment.

Count enough fields on the hemacytometer (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 sonification. The sonification energy has to be adjusted to avoid disruption of the cells.

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 should 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.

Mayamaea permitis

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.

An example of appropriate procedures of handling *Mayamaea permitis* cultures is given in the following:

M. permitis may form aggregates under certain growth conditions. This aggregation causes difficulties not only in accurately counting cells but also in maintaining exponential growth. The use of a reciprocating shaker instead of a rotary shaker tend to reduce the cell aggregation. If a reciprocating shaker is not available or if extensive cell aggregation is observed, each flask should be directly sonicated for 3 to 5 minutes at 40-50 kHz in an ultrasonic bath before taking sub-samples every 24 hours, through the pre-culture and testing periods. This sonication treatment, performed every 24 hours, is effective to maintain stable exponential growth of cultures. For this species, select the light intensity from the range of 20-120 $\mu E \cdot m^{-2}\ s^{-1}$. The type of flask cap can affect the growth in algae. For example, aluminium caps tend to promote higher growth compared to silicon caps. In such a case, levels of light intensity or initial cell density should be reduced.

© OECD (2026)

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_4Cl			15.0	0.280
$MgCl_2 \cdot 6(H_2O)$	12.16	0.0598	12.0	0.0590
$CaCl_2 \cdot 2(H_2O)$	4.41	0.0300	18.0	0.122
$MgSO_4 \cdot 7(H_2O)$	14.6	0.0592	15.0	0.0609
K_2HPO_4	1.044	0.00599		
KH_2PO_4			1.60	0.00919
$FeCl_3 \cdot 6(H_2O)$	0.160	0.000591	0.0640	0.000237
$Na_2EDTA \cdot 2(H_2O)$	0.300	0.000806	0.100	0.000269*
H_3BO_3	0.186	0.00300	0.185	0.00299
$MnCl_2 \cdot 4(H_2O)$	0.415	0.00201	0.415	0.00210
$ZnCl_2$	0.00327	0.000024	0.00300	0.0000220
$CoCl_2 \cdot 6(H_2O)$	0.00143	0.000006	0.00150	0.00000630
$Na_2MoO_4 \cdot 2(H_2O)$	0.00726	0.000030	0.00700	0.0000289
$CuCl_2.2(H_2O)$	0.000012	0.00000007	0.00001	0.00000006
pH	7.5		8.1	

© OECD (2026)

*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 tests with the diatom *Mayamaea permitis* OECD medium should be supplemented with 100 mg/L of $Na_2SiO_3 \cdot 9H_20$ to obtain a concentration of 9.89 mg Si/L. The pH should be adjusted to 7.0 ± 0.1 with 1.0 *N* HCl.

AAP medium should be supplemented with 202.4 mg/L of $Na_2SiO_3 \cdot 9H_2O$ to obtain a concentration of 20 mg Si/L in test with the diatom. The suitability of this medium for *M. permitis* has not been confirmed.

The pH of the medium is obtained at equilibrium between the carbonate system of the medium and the partial pressure of CO_2 in atmospheric air. An approximate relationship between pH at 25 °C and the molar bicarbonate concentration is:

$$pH_{eq} = 11.30 + \log[HCO_3]$$

With 15 mg $NaHCO_3$/L, pH_{eq} = 7.5 (U.S. EPA medium) and with 50 mg $NaHCO_3$/L, pH_{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

© OECD (2026)

Preparation of OECD medium

Nutrient	Concentration in stock solution
Stock solution 1: macro nutrients	
NH_4Cl	1.5 g/L
$MgCl_2 \cdot 6H_2O$	1.2 g/L
$CaCl_2 \cdot 2H_2O$	1.8 g/L
$MgSO_4 \cdot 7H_2O$	1.5 g/L
KH_2PO_4	0.16 g/L
Stock solution 2: iron	
$FeCl_3 \cdot 6H_2O$ $Na_2EDTA \cdot 2H_2O$	64 mg/L 100 mg/L
Stock solution 3: trace elements	
H_3BO_3	185 mg/L
$MnCl_2 \cdot 4H_2O$	415 mg/L
$ZnCl_2$	3 mg/L
$CoCl_2 \cdot 6H_2O$	1.5 mg/L
$CuCl_2 \cdot 2H_2O$	0.01 mg/L
$Na_2MoO_4 \cdot 2H_2O$	7 mg/L
Stock solution 4: bicarbonate	
$NaHCO_3$	50 g/L
$Na_2SiO_3 \cdot 9H_20$ for diatom species	

Sterilize the stock solutions 1 and 3 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
- 100 mg of $Na_2SiO_3 \cdot 9H_2O$ (for diatom *M. permitis*)

Make up to 1 000 mL with sterilised water.

Adjust pH to 7.0 ± 0.1 with 1.0 *N* HCl (for diatom *M. permitis*)

Allow sufficient time for equilibrating the medium with the atmospheric CO_2, if necessary, by bubbling with sterile, filtered air for some hours.

© OECD (2026)

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* $MgCl_2 \cdot 6H_2O$—6.082 g.
- *2.3* $CaCl_2 \cdot 2H_2O$—2.205 g.
- *2.4* *Micronutrient Stock Solution*—(see 3).
- *2.5* $MgSO_4 \cdot 7H_2O$—7.350 g.
- *2.6* K_2HPO_4—0.522 g.
- *2.7* $NaHCO_3$—7.500 g.
- *2.8* $Na_2SiO_3 \cdot 9H_2O$—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* $MnCl_2 \cdot 4H_2O$—207.690 mg.
- *3.3* $ZnCl_2$—1.635 mg.
- *3.4* $FeCl_3 \cdot 6H_2O$—79.880 mg.
- *3.5* $CoCl_2 \cdot 6H_2O$—0.714 mg.
- *3.6* $Na_2MoO_4 \cdot 2H_2O$—3.630 mg.
- *3.7* $CuCl_2 \cdot 2H_2O$—0.006 mg.
- $Na_2EDTA \cdot 2H_2O$—150.000 mg. [Disodium (Ethylenedinitrilo) tetraacetate].
- $Na_2SeO_4 \cdot 5H_2O$—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 □m membrane filter if a particle counter is to be used or a 0.45 □m filter if a particle counter is not to be used.

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

NOTE 3: The suitability of AAP medium has not been validated for *M. permitis.*

© OECD (2026)

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 should be established and used.

All operations should 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.

© OECD (2026)

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 should 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. Exponential growth of the inoculum used in a given test will be documented in the respective test report (see e.g. validity criteria). Daily measurements of biomass in the pre-culture prior to testing can be a useful tool to confirm exponential growth of the algal pre-culture prior to testing. When the algal cultures contain deformed or abnormal cells, they should be discarded

© OECD (2026)

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 analysis

Models

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 zero for C → 0 and one for C → 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

© OECD (2026)

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 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}.

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 should 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)

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

with a variance $Var(Y_i) = Var(r_i/r_0) \cong (\partial Yi / \partial r_i)^2 \cdot Var(r_i) + ((\partial Y_i/\partial r_0)^2 \cdot Var(r_0)$ 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: $Var(r_i) = \sigma^2/m_i$

the total variance of the relative responseY_i thus becomes $Var(Y_i) = \sigma^2/(r_0^2 \cdot m_i) + r_i^2 \cdot \sigma^2/r_0^4 \cdot m_0$

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

© OECD (2026)

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 (α, β, Concentration) and utilize the definition relationships f (α, β, EC_{10}) = 0.1 and f (α, β, EC_{50}) = 0.5 to substitute f (α, β, concentration) with an equivalent function g(EC_{10}, EC_{50}, 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_i 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.

Draper, N.R. and Smith, H. (1981). Applied Regression Analysis, second edition. Wiley, New York.
Bruce, R..D. and Versteeg,, D.J.(1992) A Statistical Procedure for Modelling Continuous Ecotoxicity Data. *Environ. Toxicol. Chem.*11, 1485-1494

Andersen, J.S., Holst, H., Spliid, H., Andersen, H., Baun, A. & Nyholm, N. (1998): Continuous ecotoxicological data evaluated relative to a control response. *Journal of Agricultural, Biological and Environmental Statistics*, 3, 405-420.

© OECD (2026)

Annex 6. Example of calculation of a coefficient of variation daily/section-by-section specific growth rates

This annex is presenting an example of calculation of mean coefficient of variation (CV) for daily/section-by-section specific growth rates, as defined in Annex 1:

1. Calculate the standard deviation (SD) of the daily/section-by-section specific growth rates over the entire test period for the respective replicate;
2. Calculate CV (%) by dividing the SD calculated under point 1 by the average specific growth rate for the respective replicate;
3. Calculate the mean value out of all values calculated under point 2 to get the mean CV (%) of the daily/section-by-section specific growth rate in replicate control cultures.

Considering the results in table 1 obtained for a 72h test:

Table 1. Cell numbers and calculated Growth rate (average specific growth rate*) at 72h

	Cell number (1.E+04 cells/mL)				Growth rate (day^{-1})
	0h	**24h**	**48h**	**72h**	**0 – 72h**
Control replicates	1.00	5.76	27.41	189.23	1.748
	1.00	6.22	23.00	187.77	1.745
	1.00	5.51	25.34	143.14	1.655
	1.00	6.23	29.93	194.84	1.757
	1.00	5.95	30.16	212.16	1.786
	1.00	5.59	23.41	113.70	1.578
Mean	***1.00***	***5.88***	***26.54***	***173.47***	***1.711***

*Use Equation [1] in paragraph 49 to calculate the average specific growth rate

The daily/section-by-section specific growth rates for the respective replicate are calculated according to paragraph 49, and, for each replicate, the CV (%) is determined (Table 2).

© OECD (2026)

Table 2. Daily/section-by-section specific growth rate and CV for individual replicate calculation

0 – 24h	24 – 48h	48 – 72h	SD***	CV of individual replicate (%)
1.751	1.560	1.932	0.186	10.647
1.828	1.308	2.100	0.402	23.061
1.707	1.526	1.731	0.112	6.783
1.829	1.569	1.873	0.164	9.344
1.783	1.623	1.951	0.164	9.176
1.721	1.432	1.580	0.144	9.153

**Use Equation [1] in paragraph 49 to calculate the daily/section-by-section growth rate

***The CV is calculated using STDEV.S, which uses the following formula:

$$\sqrt{\frac{\sum(x-\bar{x})^2}{(n-1)}}$$

Where x is the sample mean AVERAGE (number1, number2,…..) and n is the sample size.

Then the mean of the calculated CV for each replicate is determined (Table 3) and compared to the 35% threshold value defined in paragraph 12.

Table 3. Mean of individual CV calculation

CV of individual replicate (%)	Mean CV (%)
10.647	11.360
23.061	
6.783	
9.344	
9.176	
9.153	

© OECD (2026)