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

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	
$FeCl_3 \cdot 6H_2O$	64 mg/L
$Na_2EDTA \cdot 2H_2O$	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
$Na_2SiO_3 \cdot 9H_20$	

Sterilize the stock solutions by membrane filtration (mean pore diameter 0.2 µm) or by autoclaving (120 °C, 15 min). Store the solutions in the dark at 4 °C.
Do not autoclave stock solutions 2 and 4, but sterilise them by membrane filtration.

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

Add to 500 ml of sterilised water:

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

Make up to 1 000 mL with sterilised water.

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

Preparation of U.S. EPA medium

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

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

2.1 $NaNO_3$—12.750 g.
2.2 $MgCl_2 \cdot 6H_2O$—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.
3.8 $Na_2EDTA \cdot 2H_2O$—150.000 mg. [Disodium (Ethylenedinitrilo) tetraacetate].
3.9 $Na_2SeO_4 \cdot 5H_2O$—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 µm membrane filter if a particle counter is to be used or a 0.45 µm filter if a particle counter is not to be used.

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

ANNEX 4

EXAMPLE OF A PROCEDURE FOR THE CULTURING OF ALGAE

General observations

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

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

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

Equipment and materials

See under Test Guideline: Apparatus.

Procedures for obtaining algal cultures

Preparation of nutrient solutions (media):

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

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

Stock culture:

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

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

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

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

Pre-culture:

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

ANNEX 5

DATA ANALYSIS BY NONLINEAR REGRESSION

General considerations

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

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

Regression 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 one for $C \rightarrow 0$ and zero for $C \rightarrow$ infinity.

The use of continuous threshold function models (e.g. the Kooijman model "for inhibition of population growth" Kooijman et al. 1996) is a recently proposed or alternative to asymptotic models. This model assumes no effects at concentrations below a certain threshold EC_{0+} that is estimated by extrapolation of the response concentration relationship to intercept the concentration axis using a simple continuous function that is not differentiable in the starting point.

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

Procedure

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

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

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

Weighting factors

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

$$W_i = 1/Var(r_i)$$

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

Normalizing responses

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

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

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

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 response Y_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 parameters, and therefore demands more data points than non-linear regression on data that are normalized using a pre-set control response .

Inverse confidence intervals

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

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.

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