C.14. FISH JUVENILE GROWTH TEST

1. METHOD

This growth toxicity test method is a replicate of the OECD TG 215 (2000).

1.1 INTRODUCTION

This test is designed to assess the effects of prolonged exposure to chemicals on the growth of juvenile fish. It is based on a method, developed and ring-tested (1)(2) within the European Union, for assessing the effects of chemicals on the growth of juvenile rainbow trout *(Oncorynchus mykiss*) under flow-through conditions. Other well documented species may be used. For example, experience has been gained from growth tests with zebrafish (*Danio rerio*) [1] (3)(4) and ricefish (medaka, *Oryzias latipes*) (5)(6)(7).

See also General Introduction Part C.

1.2 DEFINITIONS

Lowest Observed Effect Concentration (LOEC): is the lowest tested concentration of a test substance at which the substance is observed to have a significant effect (at $p < 0.05$) when compared with the control. However, all test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC.

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

EC_x: in this Test Method is the concentration of the test substance which causes a x % variation in growth rate of the fish when compared with controls.

Loading Rate: is the wet weight of fish per volume of water.

Stocking Density: is the number of fish per volume of water.

Individual fish specific growth rate: expresses the growth rate of one individual based on its initial weight.

Tank-average specific growth rate: expresses the mean growth rate of a tank population at one concentration.

Pseudo specific growth rate: expresses the individual growth rate compared to the mean initial weight of the tank population.

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1.3 PRINCIPLE OF THE TEST METHOD

Juvenile fish in exponential growth phase are placed, after being weighted, in test chambers and are exposed to a range of sublethal concentrations of the test substance dissolved in water preferably under flow-through, or, if not possible, under appropriate semi-static (static-renewal) conditions. The test duration is 28 days. Fish are fed daily. The food ration is based on initial fish weights and may be recalculated after 14 days. At the end of the test, the fish are weighed again. Effects on growth rates are analysed using a regression model in order to estimate the concentration that would cause a x % variation in growth rate, i.e. EC_x (e.g. EC_{10}, EC_{20}, or EC_{30}). Alternatively, the data may be compared with control values in order to determine the lowest observed effect concentration (LOEC) and hence the no observed effect concentration (NOEC).

1.4 INFORMATION ON THE T EST SUBSTANCE

Results of an acute toxicity test (see Test Method C. 1.) preferably performed with the species chosen for this test, should be available. This implies that the water solubility and the vapour pressure of the test substance are known and a reliable analytical method is available for the quantification of the substance in the test solutions with known and reported accuracy and limit of detection is available.

Useful information includes the structural formula, purity of the substance, stability in water and light, pK_a, P_{ow} and results of a test for ready biodegradability (see Test Method C. 4).

1.5 VALIDITY OF THE TEST

For the test to be valid the following conditions apply:

— the mortality in the control(s) must not exceed 10 % at the end of the test;

— the mean weight of fish in the control(s) must have increased enough to permit the detection of the minimum variation of growth rate considered as significant. A ring-test (2) has shown that for rainbow trout the mean weight of fish in the controls must have increased by at least the half (i.e. 50 %) of their mean initial weight over 28 days; e.g. initial weight: 1 g/fish (= 100 %), final weight after 28 days: > 1.5 g/fish (> 150 %);

— the dissolved oxygen concentration must have been at least 60 % of the air saturation value (ASV) throughout the test;

— the water temperature must not differ by more than ± 1 °C between test chambers at any one time during the test and should be maintained within a range of 2 °C within the temperature ranges specified for the test species (Annex 1).

1.6 DESCRIPTION OF THE TEST METHOD

1.6.1 Apparatus

Normal laboratory equipment and especially the following:

a) oxygen and pH meters;

b) equipment for determination of water hardness and alkalinity;

c) adequate apparatus for temperature control and preferably continuous monitoring;

d) tanks made of chemically inert material and of suitable capacity in relation to the recommended loading and stocking density (see section 1.8.5 and Annex 1);

e) suitably accurate balance (i.e. accurate to ±0.5 %).

1.6.2 Water

Any water in which the test species shows suitable long-term survival and growth may be used as a test water. It should be of constant quality during the period of the test. The pH of the water should be within the range 6.5 to 8.5, but during a given test it should be within a range of ±0.5 pH units. Hardness above 140 mg/l (as $CaCO_3$) is recommended. In order to ensure that the dilution water will not unduly influence the test result (for example by complexion of test substance), samples should be taken at intervals for analysis. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd and Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl and SO_4), pesticides (e.g. total organophosphorus and total organochlorine pesticides), total organic carbon and suspended solids should be made, for example, every three months where a dilution water is known to be relatively constant in quality. If water quality has been demonstrated to be constant over at least one year, determinations can be less frequent and intervals extended (e.g. every 6 months). Some chemical characteristics of an acceptable dilution water are listed in Annex 2.

1.6.3 Test Solutions

Test solutions of the chosen concentrations are prepared by dilution of a stock solution.

The stock solution should preferably be prepared by simply mixing or agitating the test substance in the diluition water by using mechanical means (e.g. stirring or ultrasonication). Saturation columns (solubility columns) can be used for achieving a suitable concentrated stock solution.

The use of solvents or dispersants (solubilising agents) may be required in some cases in order to produce a suitably concentrated stock solution. Examples of suitable solvents are acetone, ethanol, methanol, dimethylsulfoxide, dimethylformamide and triethyleneglycol. Examples of suitable dispersants are Cremophor RH40, Tween 80, Methylcellulose 0.01 % and HCO-40. Care should be taken when using readily biodegradable agents (e.g. acetone) and/or highly volatile compounds as these can cause problems with bacterial built-up in flow-through tests. When a solubilising agent is used it must have no significant effects on the fish growth nor visible adverse effects on the juvenile as revealed by a solvent-only control.

For flow-through tests, a system which continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) is required to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and dilution water should be checked at intervals, preferably daily, during the test and should not vary by more than 10 % throughout the test. A ring-test (2) has shown that, for rainbow trout, a frequency of water removal during the test of 6 litres/g of fish/day is acceptable (see section 1.8.2.2).

For semi-static (renewal) tests, the frequency of medium renewal will depend on the stability of the test substance, but a daily water renewal is recommended. If, from preliminary stability tests (see section 1.4), the test substance concentration is not stable (i.e. outside the range 80-120 % of nominal or falling below 80 % of the measured initial concentration) over the renewal period, consideration should be given to the use of a flow-through test.

1.6.4 **Selection of species**

Rainbow trout (*Oncorhynchus mykiss*) is the recommended species for this test since most experience has been gained from ring-test with this species (1)(2). However, other well documented species can be used but the test procedure may have to be adapted to provide suitable test conditions. For example, experience is also available with zebrafish (*Danio rerio*) (3)(4) and ricefish (medaka, *Oryzias latipes*) (5)(6)(7). The rationale for the selection of the species and the experimental method should be reported in this case.

1.6.5 **Holding of fish**

The test fish shall be selected from a population of a single stock, preferably from the same spawning, which has been held for at least two weeks prior to the test under conditions of water quality and illumination similar to those used in the test. They should be fed a minimum ration of 2 % body weight per day and preferably 4 % body weight per day throughout the holding period and during the test.

Following a 48 h setting-in period, mortalities are recorded and the following criteria applied:

— mortalities of greater than 10 % of population in seven days: reject the entire batch;

— mortalities of between 5 % and 10 % of population: acclimation for seven additional days; if more than 5 % mortality during second seven days, reject the entire batch;

— mortalities of less than 5 % of population in seven days: accept the batch.

Fish should not receive treatment for disease in the two weeks preceding the test, or during the test.

1.7 TEST DESIGN

The 'test design' relates to the selection of the number and spacing of the test concentrations, the number of tanks at each concentration level and the number of fish per tank. Ideally, the test design should be chosen with regard to:

a) the objective of the study;

b) the method of statistical analysis that will be used;

c) the availability and cost of experimental resources.

The statement of the objective should, if possible, specify the statistical power at which a given size of difference (e.g. in growth rate) is required to be detected or, alternatively, the precision with which the EC_x (e.g. with x = 10, 20, or 30, and preferably not less than 10) is required to be estimated. Without this, a firm prescription of the size of the study cannot be given.

It is important to recognise that a design which is optimal (makes best use of resources) for use with one method of statistical analysis is not necessarily optimal for another. The recommended design for the estimation of a LOEC/NOEC would not therefore be the same as that recommended for analysis by regression.

In most of cases, regression analysis is preferable to the analysis of variance, for reasons discussed by Stephan and Rogers (8). However, when no suitable regression model is found ($r^2 < 0.9$) NOEC/LOEC should be used.

1.7.1 **Design for analysis by regression**

The important considerations in the design of a test to be analysed by regression are:

a) The effect concentration (e.g. $EC_{10,20,30}$) and the concentration range over which the effect of the test substance is of interest, should necessarily be spanned by the concentrations included in the test. The precision with which estimates of effect concentrations can be made, will be best when the effect concentration is in the middle of the range of concentrations tested. A preliminary range-finding test may be helpful in selecting appropriate test concentrations.

b) To enable satisfactory statistical modelling, the test should include at least one control tank and five additional tanks at different concentrations. Where appropriate, when a solubilising agent is used, one control containing the solubilising agent at the highest tested concentration should be run in addition to the test series (see sections 1.8.3 and 1.8.4).

c) An appropriate geometric series or logarithmic series (9) (see Annex 3) may be used. Logarithmic spacing of test concentration is to be preferred.

d) If more than six tanks are available, the additional tanks should either be used to provide replication or distributed across the range of concentrations in order to enable closer spacing of the levels. Either of these measures are equally desirable.

1.7.2 **Design for estimation of a NOEC/LOEC using Analysis of Variance (ANOVA)**

There should preferably be replicate tanks at each concentration, and statistical analysis should be at the tank level (10). Without replicate tanks, no allowance can be made for variability between tanks beyond that due to individual fish. However, experience has shown (11) that between-tank variability was very small compared with within-tank (i.e. between-fish) variability in the case examined. Therefore a relatively acceptable alternative is to perform statistical analysis at the level of individual fish.

Conventionally, at least five test concentrations in a geometric series with a factor preferably not exceeding 3.2 are used.

Generally, when tests are performed with replicate tanks, the number of replicate control tanks and therefore the number of fish should be the double of the number in each of the test concentrations, which should be of equal size (12)(13)(14). On the opposite, in absence of replicate tanks, the number of fish in the control group should be the same as the number in each test concentration.

If the ANOVA is to be based on tanks rather than individual fish (which would entail either individual marking of the fish or the use of 'pseudo' specific growth rates (see section 2.1.2)), there is a need for enough replication of tanks to enable the standard deviation of 'tanks-within-concentrations' to be determined. This means that the

degrees of freedom for error in the analysis of variance should be at least 5 (10). If only the controls are replicated, there is a danger that the error variability will be biased because it may increase with the mean value of the growth rate in question. Since growth rate is likely to decrease with increasing concentration, this will tend to lead to an overestimate of the variability.

1.8 PROCEDURE

1.8.1 **Selection and weighing of test fish**

It is important to minimise variation in weight of the fish at the beginning of the test. Suitable size ranges for the different species recommended for use in this test are given in Annex 1. For the whole batch of fish used in the test, the range in individual weights at the start of the test should ideally be kept to within ± 10 % of the arithmetic mean weight and, in any case, should not exceed 25 %. It is recommended to weight a subsample of fish before the test in order to estimate the mean weigh.

Food should be withheld from the stock population for 24 h prior to the start of the test. Fish should then be chosen at random. Using a general anaesthetic (e.g. an aqueous solution of 100 mg/l tricaine methane sulphonate (MS 222) neutralised by the addition of two parts of sodium bicarbonate per part of MS 222), fish should be weighted individually as wet weights (blotted dry) to the precision given in Annex 1. Those fish with weights within the intended range should be retained and then should be randomly distributed between the test vessels. The total wet weight of fish in each test vessel should be recorded. The use of anaesthetics likewise handling of fish (including blotting and weighing) may cause stress and injuries to the juvenile fish, in particular for those species of small size. Therefore handling of juvenile fish must be done with the utmost care to avoid stressing and injuring test animals.

The fish are weighed again on day 28 of the test (see section 1.8.6). However, if it is deemed necessary to recalculate the food ratio n, fish can be weighed again on day 14 of the test (see section 1.8.2.3). Other method as photographic method could be used to determine changes in fish size from which food rations could be adjusted.

1.8.2 **Conditions of exposure**

1.8.2.1 *Duration*

The test duration is ≥28 days.

1.8.2.2 *Loading rates and stocking densities*

It is important that the loading rate and stocking density is appropriate for the test species used (see Annex 1). If the stocking density is too high, then overcrowding stress will occur leading to reduced growth rates and possibly to disease. If it is too low, territorial behaviour may be induced which could also affect growth. In any case, the loading rate should be low enough in order that a dissolved oxygen concentration of at least 60 % ASV can be maintained without aeration. A ring-test (2) has shown that, for rainbow trout, a loading rate of 16 trouts of 3-5 g in a 40-litre volume is acceptable. Recommended frequency of water removal during the test is 6 litres/g of fish/day.

1.8.2.3 *Feeding*

The fish should be fed with an appropriate food (Annex 1) at a sufficient rate to induce acceptable growth rate. Care should be taken to avoid microbial growth and water turbidity. For rainbow trout, a rate of 4 % of their body weight per day is likely to satisfy these conditions (2)(15)(16)(17). The daily ration may be divided into two equal portions and given to the fish in two feeds per day, separated by at least 5 h. The ration is based on the initial total fish weight for each test vessel. If the fish are weighted again on day 14, the ration is then recalculated. Food should be withheld from the fish 24 h prior to weighing.

Uneaten food and fecal material should be removed from the test vessels each day by carefully cleaning the bottom of each tank using a suction.

1.8.2.4 *Light and temperature*

The photoperiod and water temperature should be appropriate for the test species (Annex 1).

1.8.3 **Test concentrations**

Normally five concentrations of the test substance are required, regardless of the test design (see section 1.7.2). Prior knowledge of the toxicity of the test substance (e.g. from an acute test and/or from range-finding studies) should help in selecting appropriate test concentrations. Justification should be given if fewer than five concentrations are used. The highest tested concentration should not exceed the substance solubility limit in water.

Where a solubilising agent is used to assist in stock solution preparation, its final concentration should not be greater than 0.1 ml/l and should preferably be the same in all test vessels (see section 1.6.3). However, every effort should be made to avoid use of such materials.

1.8.4 **Controls**

The number of dilution-water controls depends on the test design (see sections 1.7-1.7.2). If a solubilising agent is used, then the same number of solubilising-agent controls as dilution-water controls should also be included.

1.8.5 **Frequency of analytical determinations and measurements**

During the test, the concentrations of test substance are determined at regular intervals (see below).

In flow-through tests, the flow rates of diluent and toxicant stock solution should be checked at intervals, preferably daily, and should not vary by more than 10 % throughout the test. Where the test substance concentrations are expected to be within ± 20 % of the nominal values (i.e. within the range 80-120 %; see sections 1.6.2 and 1.6.3), it is recommended that, as a minimum, the highest and lowest test concentrations be analysed at the start of the test and at weekly intervals thereafter. For the test where the concentration of the test substance is not expected to remain within ± 20 % of nominal (on the basis of stability data of the test substance), it is necessary to analyse all test concentrations, but following the same regime.

In semi-static (renewal) tests where the concentration of the test substance is expected to remain within ±20 % of the nominal values, it is recommended that, as a minimum, the highest and lowest test concentrations be analysed when freshly prepared and immediately prior to renewal at the start of the study and weekly thereafter. For tests where the concentration of the test substance is not expected to remain within ± 20 % of nominal, all test concentrations must be analysed following the same regime as for more stable substances.

It is recommended that results be based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test substance in solution has been satisfactorily maintained within ± 20 % of the nominal or measured initial concentration throughout the test, then the results can be based on nominal or measured values.

Samples may need to be filtered (e.g. using a 0.45 µm pore size) or centrifuged. Centrifugation is the recommended procedure. However, if the test material does not adsorb to filters, filtration may also be acceptable.

During the test, dissolved oxygen, pH and temperature should be measured in all test vessels. Total hardness, alkalinity and salinity (if relevant) should be measured in the controls and one vessel at the highest concentration. As a minimum, dissolved oxygen and salinity (if relevant) should be measured three times (at the beginning, middle and end of the test). In semi-static tests, it is recommended that dissolved oxygen be measured more frequently, preferably before and after each water renewal or at least once a week. pH should be measured at the beginning and end of each water renewal in static renewal test and at least weekly in flow-through tests. Hardness and alkalinity should be measured once each test. Temperature should preferably be monitored continuously in at least one test vessel.

1.8.6 **Observations**

Weight: At the end of the test all surviving fish must be weighed as wet weights (blotted dry) either in groups by test vessel or individually. Weighing of animals by test vessel is preferred to individual weights which require that fish be individually marked. In the case of the measurement of individual weights for determination of individual fish specific growth rate, the marking technique selected should avoid stressing the animals (alternatives to freeze marking may be appropriate, e.g. the use of colored fine fishing line).

The fish should be examined daily during the test period and any external abnormalities (such as hemorrhage, discoloration) and abnormal behaviour noted. Any mortalities should be recorded and the dead fish removed as soon as possible. Dead fish are not replaced, the loading rate and stocking density being sufficient to avoid effects on growth through changes in number of fish per tank. However, the feeding rate will need to be adjusted.

2. DATA AND REPORTING

2.1 TREATMENT OF RESULTS

It is recommended that a statistician be involved in both the design and analysis of the test since this test method allows for considerable variation in experimental design as for example, in the number of test chambers, number of test concentrations, number of fish, etc. In view of the options available in test design, specific guidance on statistical procedure is not given here.

Growth rates should not be calculated for test vessels where the mortality exceeds 10 %. However, mortality rate should be indicated for all test concentrations.

Whichever method is used to analyse the data, the central concept is the specific growth rate r between time t_1 and time t_2. This can be defined in several ways depending on whether fish are individually marked or not or whether a tank average is required.

$$r_1 = \frac{\log_e w_2 - \log_e w_1}{t_2 - t_1} \times 100$$

$$r_2 = \frac{\overline{\log_e w_2} - \overline{\log_e w_1}}{t_2 - t_1} \times 100$$

$$r_3 = \frac{\log_e w_2 - \overline{\log_e w_1}}{t_2 - t_1} \times 100$$

where,

r_1 = individual fish specific growth rate

r_2 = tank-average specific growth rate

r_3 = 'pseudo' specific growth rate

w_1, w_2 = weights of a particular fish at times t_1 and t_2, respectively

$\log_e w_1$ = logarithm of the weight of an individual fish at the start of the study period

$\log_e w_2$ = logarithm of the weight of an individual fish at the end of the study period

$\overline{\log_e w_1}$ = average of the logarithms of the values w_1 for the fish in the tank at the start of the study period

$\overline{\log_e w_2}$ = average of the logarithms of the values w_2 for the fish in the tank at the end of the study period

t_1, t_2 = time (days) at start and end of study period

r_1, r_2, r_3 can be calculated for the 0-28 days period and, where appropriate (i.e. when measurement at day 14 has been done) for the 0-14 and 14-28 days periods.

2.1.1 **Analysis of results by regression (concentration-response modelling)**

This method of analysis fits a suitable mathematical relationship between the specific growth rate and concentration, and hence enables the estimation of the 'EC_x' i.e. any required EC value. Using this method the calculation of r for individual fish (r_1) is not necessary and instead, the analysis can be based on the tank-average value of r (r_2). This last method is preferred. It is also more appropriate in case of the use of smallest species.

The tank-average specific growth rates (r_2) should be plotted graphically against concentration, in order to inspect the concentration response relationship.

For expressing the relationship between r_2 and concentration, an appropriate model should be chosen and its choice must be supported by appropriate reasoning.

If the numbers of fish surviving in each tank are unequal, then the process of model fitting, whether simple or non-linear, should be weighted to allow for unequal sizes of groups.

The method of fitting the model must enable an estimate of, for example, the EC_{20} and of its dispersion (either standard error or confidence interval) to be derived. The graph of the fitted model should be shown in relation to the data so that the adequacy of the fit of the model can be seen (8)(18)(19)(20).

2.1.2 **Analysis of results for the estimation of the LOEC**

If the test has included replication of tanks at all concentration levels, the estimation of the LOEC could be based on an analysis of variance (ANOVA) of the tank-average specific growth rate (see section 2.1), followed by a suitable method (e.g. Dunnett's or Williams' test (12)(13)(14)(21)) of comparing the average r for each concentration with the average r for the controls to identify the lowest concentration for which this difference is significant at a 0.05 probability level. If the required assumptions for parametric methods are not met - non-normal distribution (e.g. Shapiro-Wilk's test) or heterogeneous variance (Bartlett's test), consideration should be given to transforming the data to homogenise variances prior to performing the ANOVA, or to carrying out a weighted ANOVA.

If the test has not included replication of tanks at each concentration, an ANOVA based on tanks will be insensitive or impossible. In this situation, an acceptable compromise is to base the ANOVA on the 'pseudo' specific growth rate r_3 for individual fish.

The average r_3 for each test concentration may then be compared with the average r_3 for the controls. The LOEC can then be identified as before. It must be recognised that this method provides no allowance for, nor protection against, variability between tanks, beyond that which is accounted for by the variability between individual fish. However, experience has shown (8) that between-tank variability was very small compared with within-tank (i.e. between fish) variability. If individual fish are not included in the analysis, the method of outlier identification and justification for its use must be provided.

2.2 INTERPRETATION OF RESULTS

The results should be interpreted with caution where measured toxicant concentrations in test solutions occur at levels near the detection limit of the analytical method or, in semi static tests, when the concentration of the test substance decreases between freshly prepared solution and before renewal.

2.3 TEST REPORT

The test report must include the following information:

2.3.1 **Test substance:**

— physical nature and relevant physical-chemical properties;

— chemical identification data including purity and analytical method for quantification of the test substance where appropriate.

2.3.2 **Test species:**

— scientific name, possibly

— strain, size, supplier, any pre-treatment, etc.

2.3.3 **Test conditions:**

— test procedure used (e.g. semi-static/renewal, flow-through, loading, stocking density, etc.);

— test design (e.g. number of test vessels, test concentrations and replicates, number of fish per vessel);

— method of preparation of stock solutions and frequency of renewal (the solubilising agent and its concentration must be given, when used);

— the nominal test concentrations, the means of the measured values and their standard deviations in the test vessels and the method by which these were attained and evidence that the measurements refer to the concentrations of the test substance in true solution;

— dilution water characteristics: pH, hardness, alkalinity, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), total organic carbon, suspended solids, salinity of the test medium (if measured) and any other measurements made;

— water quality within test vessels: pH, hardness, temperature and dissolved oxygen concentration;

— detailed information on feeding, (e.g. type of food(s), source, amount given and frequency).

2.3.4 **Results:**

— evidence that controls met the validity criterion for survival, and data on mortalities occurring in any of the test concentrations;

— statistical analytical techniques used, statistics based on replicates or fish, treatment of data and justification of techniques used;

— tabulated data on individual and mean fish weights on days 0, 14 (if measured) and 28 values of tank-average or pseudo specific growth rates (as appropriate) for the periods 0-28 days or possibly 0-14 and 14-28;

— results of the statistical analysis (i.e. regression analysis or ANOVA) preferably in tabular and graphical form and the LOEC ($p = 0.05$) and the NOEC or EC_x with, when possible, standard errors, as appropriate;

— incidence of any unusual reactions by the fish and any visible effects produced by the test substance.

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ANNEX 1

FISH SPECIES RECOMMENDED FOR TESTING AND SUITABLE TEST CONDITIONS

Species	Recommended test temperature range (°C)	Photoperiod (hours)	Recommended range for initial fish weight (g)	Required measurement precision	Loading rate (g/l)	Stocking density (per litre)	Food	Test duration (days)
Recommended species:								
Oncorhynchus mykiss rainbow trout	12.5 – 16.0	12 – 16	1 – 5	to nearest 100 mg	1.2 – 2.0	4	Dry propietary salmonid fry food	≥ 28
Other well documented species:								
Danio rerio zebrafish	21 – 25	12 – 16	0.050 – 0.100	to nearest 1 mg	0.2 – 1.0	5 – 10	Live food (*Brachionus Artemia*)	≥ 28
Oryzias latipes ricefish (Medaka)	21 – 25	12 – 16	0.050 – 0.100	to nearest 1 mg	0.2 – 1.0	5 – 20	Live food (*Brachionus Artemia*)	≥ 28

ANNEX 2

SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

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

ANNEX 3

LOGARITHMIC SERIES OF CONCENTRATIONS SUITABLE FOR TOXICITY TEST (9)

Column (Number of concentrations between 100 and 10, or between 10 and 1)*						
1	**2**	**3**	**4**	**5**	**6**	**7**
100	100	100	100	100	100	100
32	46	56	63	68	72	75
10	22	32	40	46	52	56
3.2	10	18	25	32	37	42
1.0	4.6	10	16	22	27	32
	2.2	5.6	10	15	19	24
	1.0	3.2	6.3	10	14	18
		1.8	4.0	6.8	10	13
		1.0	2.5	4.6	7.2	10
			1.6	3.2	5.2	7.5
			1.0	2.2	3.7	5.6
				1.5	2.7	4.2
				1.0	1.9	3.2
					1.4	2.4
					1.0	1.8
						1.3
						1.0

* A series of five (or more) successive concentrations may be chosen from a column. Mid-points between concentrations in column (x) are found in column (2x + 1). The values listed can represent concentrations expressed as percentage per volume or weight (mg/l or µg/l). Values can be multiplied or divided by any power of 10 as appropriate. Column 1 might be used if there was considerable uncertainty on the toxicity level.