

## OECD GUIDELINES FOR THE TESTING OF CHEMICALS

### Sediment-Water Chironomid Toxicity Test Using Spiked Water

#### INTRODUCTION

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

2. The exposure scenario used in this guideline is water spiking. The selection of the appropriate exposure scenario depends on the intended application of the test. The water exposure scenario, involving spiking of the water column, is intended to simulate a pesticide spray drift event and covers the initial peak of concentrations in pore water. It is also useful for other types of exposure (including chemical spills) except accumulation processes lasting longer than the test period.

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

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

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

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

- the use of formulated sediment allows for comparisons of toxicity and ranking substances accordingly: toxicity data from tests with natural and artificial sediments were comparable for several chemicals (2).

6. Definitions used are given in Annex 1.

### **PRINCIPLE OF THE TEST**

7. First instar chironomid larvae are exposed to a concentration range of the test chemical in sediment-water systems. The test starts by placing first instar larvae into the test beakers containing the sediment-water system and subsequently spiking the test substance into the water. Chironomid emergence and development rate is measured at the end of the test. Larval survival and weight may also be measured after 10 days if required (using additional replicates as appropriate). These data are analysed either by using a regression model in order to estimate the concentration that would cause x % reduction in emergence, larvae survival or growth (e.g. EC<sub>15</sub>, EC<sub>50</sub>, etc.), or by using statistical hypothesis testing to determine a NOEC/LOEC. The latter requires comparison of effect values with control values using statistical tests.

### **INFORMATION ON THE TEST SUBSTANCE**

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

### **REFERENCE SUBSTANCES**

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

### **VALIDITY OF THE TEST**

10. For the test to be valid the following conditions apply:
- the emergence in the controls must be at least 70% at the end of the test. (1)(6);
  - *C. riparius* and *C. yoshimatsui* emergence to adults from control vessels should occur between 12 and 23 days after their insertion into the vessels; for *C. tentans*, a period of 20 to 65 days is necessary.
  - at the end of the test, pH and the dissolved oxygen concentration should be measured in each vessel. The oxygen concentration should be at least 60 per cent of the air saturation value (ASV) at the temperature used, and the pH of overlying water should be in the 6-9 range in all test vessels;

- the water temperature should not differ by more than  $\pm 1.0$  °C. The water temperature could be controlled by isothermal room and in that case the room temperature should be confirmed in an appropriate time intervals.

## **DESCRIPTION OF THE METHOD**

### **Test vessels**

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

### **Selection of species**

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

### **Sediment**

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

- (a) 4-5 % (dry weight) peat: as close to pH 5.5 to 6.0 as possible; it is important to use peat in powder form, finely ground (particle size  $\leq 1$  mm) and only air dried.
- (b) 20 % (dry weight) kaolin clay (kaolinite content preferably above 30 %).
- (c) 75-76 % (dry weight) quartz sand (fine sand should predominate with more than 50 per cent of the particles between 50 and 200  $\mu$ m).
- (d) Deionised water is added to obtain moisture of the final mixture in a range of 30–50 %.
- (e) Calcium carbonate of chemically pure quality (CaCO<sub>3</sub>) is added adjust the pH of the final mixture of the sediment to  $7.0 \pm 0.5$ .
- (f) Organic carbon content of the final mixture should be 2 % ( $\pm 0.5$  %) and is to be adjusted by the use of appropriate amounts of peat and sand, according to (a) and (c).

14. The source of peat, kaolin clay and sand should be known. The sediment components should be checked for the absence of chemical contamination (e.g. heavy metals, organochlorine compounds, organophosphorous compounds, etc.). An example for the preparation of the formulated sediment is

described in Annex 3. Mixing of dry constituents is also acceptable if it is demonstrated that after addition of overlying water a separation of sediment constituents (e.g. floating of peat particles) does not occur, and that the peat or the sediment is sufficiently conditioned.

### Water

15. Any water which conforms to the chemical characteristics of acceptable dilution water as listed in Annexes 2 and 4 is suitable as test water. Any suitable water, natural water (surface or ground water), reconstituted water (see Annex 2) or dechlorinated tap water are acceptable as culturing water and test water if chironomids will survive in it for the duration of the culturing and testing without showing signs of stress. At the start of the test, the pH of the test water should be between 6 and 9 and the total hardness not higher than 400 mg/l as CaCO<sub>3</sub>. However, if there is an interaction suspected between hardness ions and the test substance, lower hardness water should be used (and thus, Elendt Medium M4 must not be used in this situation). The same type of water should be used throughout the whole study. The water quality characteristics listed in Annex 4 should be measured at least twice a year or when it is suspected that these characteristics may have changed significantly.

### Stock solutions - Spiked water

16. Test concentrations are calculated on the basis of water column concentrations, i.e. the water overlying the sediment. Test solutions of the chosen concentrations are usually prepared by dilution of a stock solution. Stock solutions should preferably be prepared by dissolving the test substance in test medium. The use of solvents or dispersants may be required in some cases in order to produce a suitably concentrated stock solution. Examples of suitable solvents are acetone, ethanol, methanol, ethylene glycol monoethyl ether, ethylene glycol dimethyl ether, dimethylformamide and triethylene glycol. Dispersants which may be used are Cremophor RH40, Tween 80, methylcellulose 0.01% and HCO-40. The solubilising agent concentration in the final test medium should be minimal (i.e.  $\leq 0.1$  ml/l) and should be the same in all treatments. When a solubilising agent is used, it must have no significant effects on survival nor visible adverse effect on the chironomid larvae as revealed by a solvent-only control. However, every effort should be made to avoid the use of such materials.

## TEST DESIGN

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

### Design for analysis by regression

18. The effect concentration (e.g. EC<sub>15</sub>, EC<sub>50</sub>) and the concentration range, over which the effect of the test substance is of interest, should be spanned by the concentrations included in the test. Generally, the accuracy and especially validity, with which estimates of effect concentrations (EC<sub>x</sub>) can be made, is improved when the effect concentration is within the range of concentrations tested. Extrapolation much below the lowest positive concentration or above the highest concentration should be avoided. A preliminary range-finding test is helpful for selecting the range of concentrations to be used (see paragraph 27).

19. If the  $EC_x$  is to be estimated, at least five concentrations and three replicates for each concentration should be tested. In any case, it is advisable that sufficient test concentrations are used to allow a good model estimation. The factor between concentrations should not be greater than two (an exception could be made in cases when the dose response curve has a shallow slope). The number of replicates at each treatment can be reduced if the number of test concentrations with different responses is increased. Increasing the number of replicates or reducing the size of the test concentration intervals tends to lead to narrower confidence intervals for the test. Additional replicates are required if 10-day larval survival and growth are to be estimated.

#### **Design for estimation of a NOEC/LOEC**

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

#### **Limit test**

21. A limit test may be performed (one test concentration and control) if no effects were seen in the preliminary range-finding test. The purpose of the limit test is to indicate that the toxic value of the test substance is greater than the limit concentration tested. No suggestion for a recommended concentration can be made in this guideline; this is left to the regulators' judgement. Usually, at least six replicates for both the treatment and control are necessary. Adequate statistical power to detect a 20 % difference from the control at the 5 % level of significance ( $p = 0.05$ ) should be demonstrated. With metric response (development rate and weight), the t-test is a suitable statistical method if data meet the requirements of this test (normality, homogeneous variances). The unequal-variance t-test or a non parametric test, such as the Wilcoxon-Mann-Whitney test may be used, if these requirements are not fulfilled. With the emergence ratio, the Fisher exact test is appropriate.

### **PROCEDURE**

#### **Conditions of exposure**

##### **Preparation of spiked water-sediment system**

22. Appropriate amounts of formulated sediment (see paragraphs 13-14 and Annex 3) are added in the test vessels to form a layer of at least 1.5 cm. Water is added to a depth of 6 cm (see paragraph 15). The ratio of the depth of the sediment layer and the depth of the water should not exceed 1:4 and the sediment layer should not be deeper than 3 cm. The sediment-water system should be left under gentle aeration for seven days prior to addition of test organisms (see paragraph 14 and Annex 3). To avoid separation of sediment ingredients and re-suspension of fine material during addition of test water in the water column, the sediment can be covered with a plastic disc while water is poured onto it, and the disc is removed immediately afterwards. Other devices may also be appropriate.

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

### Addition of test organisms

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

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

26. Twenty-four hours after adding the larvae, the test substance is spiked into the overlying water column, and slight aeration is again supplied. Small volumes of test substance solutions are applied below the surface of the water using a pipette. The overlying water should then be mixed with care not to disturb the sediment.

### Test concentrations

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

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

### Controls

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

### Test system

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

## Food

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

## Incubation conditions

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

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

## Exposure duration

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

## Observations

### Emergence

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

36. The test vessels should be observed at least three times per week to make visual assessment of any abnormal behaviour (e.g. leaving sediment, unusual swimming), compared with the control. During the period of expected emergence a daily count of emerged midges is necessary. The sex and number of fully emerged midges are recorded daily. After identification the midges are removed from the vessels. Any egg masses deposited prior to the termination of the test should be recorded and then removed to prevent re-introduction of larvae into the sediment. The number of visible pupae that have failed to emerge is also recorded. Guidance on measurement of emergence is provided in Annex 5.

**Growth and survival**

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

**Analytical measurements****Concentration of the test substance**

38. As a minimum, samples of the overlying water, the pore water and the sediment must be analysed at the start (preferably one hour after application of test substance) and at the end of the test, at the highest concentration and a lower one. These determinations of test substance concentration inform on the behaviour/partitioning of the tested chemical in the water-sediment system. Sampling of sediment at the start of the test may influence the test system (e.g. removing test larvae), thus additional test vessels should be used to perform analytical determinations at the start and during the test if appropriate (see paragraph 39). Measurements in sediment might not be necessary if the partitioning of the test substance between water and sediment has been clearly determined in a water/sediment study under comparable conditions (e.g. sediment to water ratio, type of application, organic carbon content of sediment).

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

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

**Physical-chemical parameters**

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

**DATA AND REPORTING****Treatment of results**

42. The purpose of this test is to determine the effect of the test substance on the development rate and the total number of fully emerged male and female midges, or in the case of the 10-day test effects on survival and weight of the larvae. If there are no indications of statistically different sensitivities of sexes, male and female results may be pooled for statistical analyses. The sensitivity differences between sexes



can be statistically judged by, for example, a  $\chi^2$ -r  $\times$  2 table test. Larval survival and mean individual dry weight per vessel must be determined after 10 days where required.

43. Effect concentrations expressed as concentrations in the overlaying water, are calculated preferably based on measured concentrations at the beginning of the test (see paragraph 38).

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

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

### Emergence ratio

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

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

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

where:

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

47. An alternative that is most appropriate for large sample sizes, when there is extra binomial variance, is to treat the emergence ratio as a continuous response and use procedures such as William's test when a monotonic dose-response is expected and is consistent with these ER data. Dunnett's test would be appropriate where monotonicity does not hold. A large sample size is defined here as the number emerged and the number not emerging both exceeding five, on a per replicate (vessel) basis.

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

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

### Development rate

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

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

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

where:

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

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

where:

- $\text{day}_i$  : inspection day (days since application)
- $l_i$  : length of inspection interval  $i$  (days, usually 1 day)

### Test report

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

Test substance:

- physical nature and, where relevant, physical-chemical properties (water solubility, vapour pressure, partition coefficient in soil (or in sediment if available), stability in water, etc.);

- chemical identification data (common name, chemical name, structural formula, CAS number, etc.) including purity and analytical method for quantification of test substance.

Test species:

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

Test conditions:

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

Results:

- the nominal test concentrations, the measured test concentrations and the results of all analyses to determine the concentration of the test substance in the test vessel;
- water quality within the test vessels, i.e. pH, temperature, dissolved oxygen, hardness and ammonia;
- replacement of evaporated test water, if any;
- number of emerged male and female midges per vessel and per day;
- number of larvae which failed to emerge as midges per vessel;
- mean individual dry weight of larvae per vessel, and per instar, if appropriate;
- percent emergence per replicate and test concentration (male and female midges pooled);
- mean development rate of fully emerged midges per replicate and treatment rate (male and female midges pooled);
- estimates of toxic endpoints for example  $EC_x$  (and associated confidence intervals), NOEC and/or LOEC, and the statistical methods used for their determination;
- discussion of the results, including any influence on the outcome of the test resulting from deviations from this Guideline.

**LITERATURE**

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ANNEX 1DEFINITIONS

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

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

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

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

Spiked water is the test water to which test substance has been added.

ANNEX 2RECOMMENDATIONS FOR CULTURE OF *CHIRONOMUS RIPARIUS*

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

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

Dilution water

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

Feeding larvae

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

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

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

Feeding emerged adults

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

Emergence

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

#### Egg masses

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

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

#### Set-up of new culture vessels

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

#### Preparation of test solutions "M4" and "M7"

12. Elendt (1990) has described the "M4" medium. The "M7" medium is prepared as the "M4" medium except for the substances indicated in Table 1, for which concentrations are four times lower in "M7" than in "M4". A publication on the "M7" medium is in preparation (Elendt, personal communication). The test solution should not be prepared according to Elendt and Bias (1990) for the concentrations of  $\text{NaSiO}_3$ ,  $5 \text{ H}_2\text{O}$ ,  $\text{NaNO}_3$ ,  $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$  given for the preparation of the stock solutions are not adequate.

#### Preparation of the "M7"-medium

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

#### Reference

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

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

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

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

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

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

**Table 3: Vitamin stock solution for medium M4 and M7**

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

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

### **References**

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ANNEX 3PREPARATION OF FORMULATED SEDIMENTSediment composition

The composition of the formulated sediment should be as follows:

Constituent	Characteristics	% of sediment dry weight
Peat	Sphagnum moss peat, as close to pH 5.5-6.0 as possible, no visible plant remains, finely ground (particle size $\leq 1$ mm) and air dried	4-5
Quartz sand	Grain size: > 50% of the particles should be in the range of 50-200 $\mu\text{m}$	75-76
Kaolinite clay	Kaolinite content $\geq 30\%$	20
Organic carbon	Adjusted by addition of peat and sand	2 ( $\pm 0.5$ )
Calcium carbonate	$\text{CaCO}_3$ , pulverised, chemically pure	0.05 – 0.1
Water	Conductivity $\leq 10 \mu\text{S/cm}$	30 – 50

Preparation

The peat is air dried and ground to a fine powder. A suspension of the required amount of peat powder in deionised water is prepared using a high-performance homogenising device. The pH of this suspension is adjusted to  $5.5 \pm 0.5$  with  $\text{CaCO}_3$ . The suspension is conditioned for at least two days with gentle stirring at  $20 \pm 2$  °C, to stabilise pH and establish a stable microbial component. pH is measured again and should be  $6.0 \pm 0.5$ . Then the peat suspension is mixed with the other constituents (sand and kaolin clay) and deionised water to obtain an homogeneous sediment with a water content in a range of 30–50 per cent of dry weight of the sediment. The pH of the final mixture is measured once again and is adjusted to 6.5 to 7.5 with  $\text{CaCO}_3$  if necessary. Samples of the sediment are taken to determine the dry weight and the organic carbon content. Then, before it is used in the chironomid toxicity test, it is recommended that the formulated sediment be conditioned for seven days under the same conditions which prevail in the subsequent test.

Storage

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

References

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ANNEX 4**CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER**

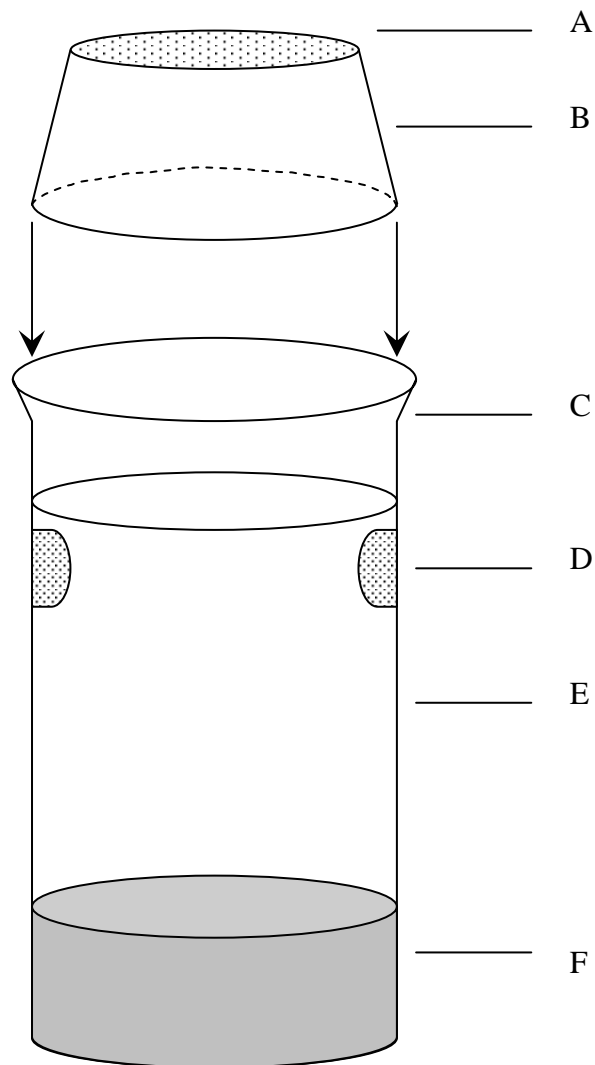
<b>SUBSTANCE</b>	<b>CONCENTRATIONS</b>
Particulate matter	< 20 mg/l
Total organic carbon	< 2 mg/l
Unionised ammonia	< 1 µg/l
Hardness as CaCO <sub>3</sub>	<400 mg/l*
Residual chlorine	< 10 µg/l
Total organophosphorus pesticides	< 50 ng/l
Total organochlorine pesticides plus polychlorinated biphenyls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

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

ANNEX 5

GUIDANCE FOR MONITORING EMERGENCE OF CHIRONOMID LARVAE

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



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

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