

## **OECD GUIDELINE FOR THE TESTING OF CHEMICALS**

### **Chironomus sp., Acute Immobilisation Test**

#### **INTRODUCTION**

1. This Test Guideline (TG) describes an acute toxicity test to assess effects of chemicals towards chironomids. As a water-only chironomid acute immobilisation assay, it is designed to complement the TGs 218, 219 and 233 for chironomid chronic toxicity assays (10) (11) (14).
2. The methodology is based on TG 202: *Daphnia* sp., Acute Immobilisation Test (12). Also, information from existing long-term sediment toxicity test protocols for *C. riparius* and *C. dilutus* developed in Europe and North America (6) (5) (18) (19) (2) (20) and included in previous ring-test experiments (17) (8) (20) is taken into account.

#### **PRINCIPLE OF THE TEST**

3. First instar *Chironomus* sp. larvae are exposed to a range of concentrations of the test substance in water-only vessels for a period of 48 hours. Immobilisation is recorded at 24 and 48 hours. The EC<sub>50</sub> is calculated at 24 and 48 hours (if data allow). Definitions/Descriptions are set out in Annex 1.

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

4. The water solubility and the vapour pressure of the test substance should be known and a reliable analytical method for the quantification of the substance in the test solutions, with reported recovery efficiency and limit of determination, should be available. Useful information on the test substance includes the structural formula, purity, stability in water and light, octanol-water partition coefficient K<sub>ow</sub> and results of a test for ready biodegradability (TG 301 or TG 310). The physical appearance of the test substance should be described. Guidance for testing substances with physical chemical properties that make them difficult to test is provided in the OECD guidance document No. 23 (9).

#### **REFERENCE SUBSTANCES**

5. A reference substance may be tested regularly as a means of assuring that the test system and conditions are reliable. Toxicants which have been used in international ring-tests and validation studies are recommended for this purpose. A ring test was performed with the acute chironomid test, using 3,5-dichlorophenol and potassium chloride as test substances (see Annex 4) (15) (21). Other examples of reference toxicants used successfully with *Chironomus* sp. are: lindane, pentachlorophenol, cadmium chloride (5) (17) (1) (2) (20). Test(s) with a reference substance should be conducted as necessary, for

#### **© OECD, (2011)**

You are free to use this material for personal, non-commercial purposes without seeking prior consent from the OECD, provided the source is duly mentioned. Any commercial use of this material is subject to written permission from the OECD.

example after introducing new stock organisms into the culture or after significant changes in culture conditions. However, at least two reference tests per year should be conducted.

### VALIDITY OF THE TEST

6. For a test to be valid, the following performance criteria apply:

- In the control, including the solvent control if appropriate, not more than 15 percent of the larvae should show immobilisation or other signs of disease or other stress (e.g. abnormal appearance or unusual behaviour, such as trapping at the water surface) at the end of the test.
- The dissolved oxygen concentration at the end of the test should be  $\geq 3$  mg/L in control and test vessels.

### DESCRIPTION OF THE METHOD

#### *Apparatus*

7. Test vessels and other apparatus that will come into contact with the test solutions should be made entirely of glass or other chemically inert material. Suitable test vessels are for example 50 ml Petri dishes (height: 3 cm, inner diameter: 4.7 cm) or 100 mL beakers (height: 7 cm, inner diameter: 4.5 cm), which should be cleaned before each use using standard laboratory procedures. Test vessels should be loosely covered (e.g. with a glass lid, watch glass or parafilm) to reduce the loss of water due to evaporation and to avoid the entry of dust into the solutions. Volatile and other test substances, which might be difficult to test, should be handled according to the recommendations described in the OECD guidance document No. 23 (9).

8. In addition, some or all of the following equipment may be used: oxygen-meter (with microelectrode or other suitable equipment for measuring dissolved oxygen in low volume samples); pH-meter; equipment for the determination of hardness; adequate apparatus for temperature control, etc. If the dilution water is from a surface or groundwater source, equipment for the determination of the total organic carbon (TOC) concentration and/or equipment for the determination of the chemical oxygen demand (COD) will be necessary.

#### *Test Organisms*

9. First instar larvae of *Chironomus riparius* are used in this test, since they have been shown to be the most sensitive larval stage (21). Further, this instar is free swimming and therefore not stressed by the absence of sediment. Although *C. riparius* is the preferred species, *C. dilutus* or *C. yoshimatsui* may also be used. Details on culturing methods are available for *C. riparius* (Annex 2), *C. dilutus* (20) and *C. yoshimatsui* (18). Test organisms should come from a source (preferably in-house culture) where identity of the test species has been confirmed.

10. The larvae should be derived from a healthy stock (i.e. showing no signs of stress such as high mortality, discoloured animals, etc.) with a known history (breeding method, culture conditions). All organisms used for an individual test should have originated from the same culture. The cultures should be maintained in conditions (light, temperature, and medium) similar to those to be used in the test. If the *Chironomus* sp. culture medium to be used in the test is different from that used for routine *Chironomus* sp. culture, it is good practice to include a pre-test acclimation period by placing egg masses for hatching

and maintaining the first instar larvae in test dilution water at test temperature until the start of the exposure.

### *Holding and dilution water*

11. Natural water (surface or groundwater), reconstituted water or dechlorinated tap water are acceptable as holding and dilution water if *Chironomus* sp. survive in it during culture, acclimation and testing without showing signs of stress. Any water which conforms to the chemical characteristics of acceptable dilution water as listed in [Annex 3](#) is suitable as a test water. It should be of constant quality during the period of the test. Reconstituted water can be made up by adding specific amounts of reagents of recognised analytical grade to deionised or distilled water. Examples of reconstituted water are given in [Annex 2](#).

12. In case of natural water, the water quality characteristics listed in [Annex 3](#) should be measured at least twice a year or when it is suspected that these characteristics may have changed significantly. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni) should also be made. If dechlorinated tap water is used, chlorine analysis is desirable. If the dilution water is from a natural source, conductivity and total organic carbon (TOC) or chemical oxygen demand (COD) should be measured.

13. To avoid the necessity of adaptation prior to the test, it is recommended that the water used in the test is the same as used for culturing. The dilution water may be aerated prior to use for the test so that the dissolved oxygen concentration has reached saturation.

### *Test Solutions*

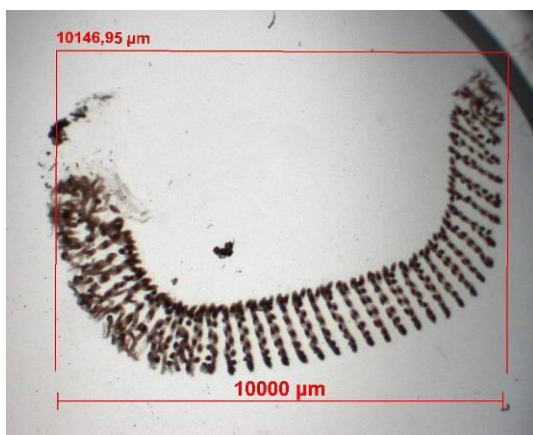
14. Test solutions of the chosen concentrations are usually prepared by dilution of a stock solution. Stock solutions should preferably be prepared by dissolving the test substance directly in the dilution water. As far as possible, the use of solvents or dispersants should be avoided; however, use of solvents may be necessary in some cases in order to produce a suitably concentrated and homogeneous stock solution. Guidance for suitable solvents as well as other aspects of handling difficult substances, such as biodegrading-, complexing-, ionising- or multi-component substances and mixtures is available (9). For active ingredients or other single chemicals (i.e. not mixtures), the test substance in the test solutions should not exceed the practical limit of solubility in the dilution water. If solubilising agents are used, the concentration of solvent should be equal for all test concentrations and in the solvent control. In addition, the maximum solvent concentration should be 100 µL/L or 100 mg/L, whichever is lowest (9).

15. The test should generally be conducted without adjustment of pH. If the pH does not remain in the range 6-9, then a second test should be conducted, adjusting the pH of the stock solution to that of the dilution water before preparing the test solutions. The pH adjustment, preferably with 0.1 M HCl and 0.1 M NaOH, should be made in such a way that the stock solution concentration is not changed to any significant extent and that no chemical reaction, such as precipitation of the test substance, is caused. In order to maintain test concentrations for 48 hours, pH adjustments within the pH range 6-9 may be appropriate for chemicals that may be unstable over this pH range.

## PROCEDURE

### *Preparation of Test Organisms Prior to Exposure*

16. Four to five days before adding 1<sup>st</sup> instar larvae to the test vessels (test initiation), fresh egg masses (< 24 h) should be taken from the culture. To ensure the correct age of the egg masses used in the test, the rims of the culture vessels should be cleaned from old egg masses 24 h before collection (the old egg masses can stay in the culture vessels). For starting the toxicity test, at least three but preferably six egg ropes are selected from the culture and they are allowed to hatch together with some added food (see paragraph 17). Larvae for the test are randomly selected from this batch. Aged medium from the stock culture or freshly prepared medium may be used. Normally, the larvae begin to hatch within a few days after the eggs are laid (2 to 3 days for *C. riparius* at 20°C, and 1 to 4 days for *C. dilutus* at 23°C and for *C. yoshimatsui* at 25°C). First instar larvae should be used in the test. If considered necessary, the instar of midge larvae can be checked by measuring the head capsule width (20). Figures 1 and 2 show a freshly laid egg mass and an egg mass that has almost completed hatching, respectively.



**Figure 1:** A freshly (< 24 h) laid egg mass of *C. riparius* (21)



**Figure 2:** First-instar larvae from an almost completely hatched egg mass of *C. riparius* (21)

17. Larvae should not be fed during the test. However, feeding prior to exposure (i.e. directly after hatching) is important in order to ensure  $\geq 85\%$  survival of the larvae in the controls at the end of the exposure period. A few droplets of filtrate from finely ground suspension of flaked fish-food (e.g. Tetra-Min or Tetra-Phyll, see also (11) in the amount of 0.05-0.5 mg per larva should be sufficient for young larvae. Green algae have also been suggested as a food source (10) (11), but care should be taken when larvae are added to the test vessels not to “inoculate” the test medium with algae, which may influence test compound availability. As an alternative to flaked fish-food, plant material may be used, for example

stinging nettle (*Urtica dioica*), mulberry (*Morus alba*), white clover (*Trifolium repens*) or spinach (*Spinacia oleracea*). Other plant material (Cerophyl or alpha-cellulose) may also be used. However, as the amounts of food recommended in the literature vary considerably, it is recommended that individual laboratories determine the amount of food required prior to exposure to ensure adequate survival in the test. Fish food extract, which can be prepared by boiling the fish food suspension followed by removal of the larger particles, e.g. by filtration or by discarding the settled fraction, would typically have around 3.7 g TOC/L of which very small amounts would be carried over to the test vessels.

18. Chironomid larvae may get trapped at the water surface and may not be able to move back into the water column. This mostly depends on the physico-chemical properties of the test substance and/or test medium, but can also be related to the cleaning process of the glass vessels. Introduction of the larvae into the test system by releasing them below the water surface is one way to reduce trapping. In case of trapped larvae a small droplet of treatment medium dropped gently from a pipette on their body may be able to sink them below the water surface. The trapping phenomenon may result in invalid studies (i.e. high control mortality). A possible solution to avoid trapping is the addition of a tiny amount of a dispersant, e.g. Tween 80 at 2 µL/L, to reduce the surface tension of the medium, although the use of dispersants is not generally advocated (see also paragraph 14). Another way might be to physically keep the individuals from getting to the air/water interface, e.g. by using sealed vessels without airspace or using a mesh to retain organisms below the surface.

### ***Conditions of exposure***

#### *Test groups and controls*

19. Test vessels should be filled with appropriate volumes of test solutions. *Chironomus* sp. first instar larvae should be randomly allocated to the test vessels using a blunt pipette. At least 20 animals, preferably divided into four groups of five animals each, should be used for each test concentration and controls. At least 2 mL of test solution should be provided for each animal (i.e. at least a volume of 10 mL for five larvae per test vessel; the volume should also be sufficient to allow for adequate samples to be taken at the end of the test for analytical determination of the exposure concentrations).

#### *Test concentrations*

20. A range-finding test may be conducted to determine the range of concentrations for the definitive test. For this purpose, the larvae are exposed to a series of widely spaced concentrations of the test substance. At least ten larvae should be exposed divided over two replicates of each test concentration for 48 hours.

21. In the definitive test, at least five test concentrations should be used, with a dilution water control and solvent control (if appropriate). The solvent control contains the solvent in a concentration identical to the level used in treatments. Concentrations should be arranged in a geometric series with a spacing factor preferably not exceeding 2.2. Justification should be provided if fewer than five concentrations or a larger spacing factor is used, e.g. a shallow slope of the concentration-response curve. The highest test concentration should preferably result in 100% immobilisation and the lowest test concentration should preferably give no observable effect. However, these effect levels are secondary to adequately defining the EC<sub>50</sub>.

#### *Incubation conditions*

22. The water temperature should be within the range of 18 to 22°C for *C. riparius*. For *C. dilutus* and *C. yoshimatsui*, recommended temperatures are 23°C and 25°C, respectively, and for each individual

test, the temperature should be constant within  $\pm 1^\circ\text{C}$ . A 16-hour light and 8-hour dark cycle is recommended and the light intensity should be between 500 to 1000 lux. Complete darkness is also acceptable, especially for test substances that are unstable in light. The test vessels should be positioned in a randomized way.

#### *Duration*

23. The exposure commences with the addition of larvae to the test solutions and lasts for 48 h.

#### *Observations*

24. Each test vessel should be checked for immobilised larvae at 24 and 48 hours after the beginning of the exposure. To aid observations, a stereo microscope or light table may be used. Those animals that are not able to change their position (by crawling or swimming movements) within 15 seconds after mechanical stimulation, e.g. by subjecting the larvae to a gentle stream of water from a Pasteur pipette or agitation of the test vessel, are considered to be immobilised. The movements of larvae are erratic and phases of high activity (i.e. swimming by quickly whipping their body into an alpha shape when most contracted and then inside out again) are interrupted by phases of no movement. Immobility is used as surrogate parameter for lethality, similar to the definition for neonate daphnids in the 48-h acute Test Guideline (12), since it is difficult to establish mortality in first instar larvae. In addition to immobility, any abnormal behaviour or unusual appearance should be reported. Immobilized larvae observed at 24 hours are not removed from the test vessels so that their immobility may be confirmed at test end. Missing larvae are counted as immobile.

#### *Analytical Measurements*

25. The dissolved oxygen and pH are measured, as a minimum, in the control(s) and in the highest test substance concentration at the beginning and end of the test. The dissolved oxygen concentration in the control(s) should be in compliance with the validity criterion. The oxygen level in control and test vessels should be reported in mg/L. The pH should normally not vary by more than 1.5 units over the course of the test. Temperature is usually measured in control vessels and it should be recorded preferably continuously throughout the test or, as a minimum, at the beginning and end of the test.

26. The concentration of the test substance should be measured, as a minimum, in the control(s), the highest and lowest test concentration, but preferably in all treatments, at the beginning and end of the test. It is recommended that results be based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test substance has been satisfactorily maintained within  $\pm 20$  per cent of the nominal or measured initial concentration throughout the test, then the results can be based on nominal or measured initial values.

#### **LIMIT TEST**

27. A limit test with a single concentration may be performed at 100 mg/L of test substance or up to the practical limit of solubility in the test medium (whichever is lowest) in order to demonstrate that the  $\text{EC}_{50}$  is greater than this concentration. The limit test should be performed using 20 larvae (preferably divided into four groups of five), with the same number in the control(s). If the percentage of immobilisation is  $> 15\%$  at the end of the test, a full concentration-response study should be conducted. Any observed abnormal behaviour should be recorded.

## DATA AND REPORTING

### *Data*

28. Data should be summarised in tabular form, showing for each replicate of each treatment, the number of larvae exposed, and immobilisation at each observation. The percentages immobilised at 24 and 48 h are plotted against test concentrations. Data are analysed by appropriate models (e.g. probit analysis) as recommended in the statistical guidance for ecotoxicity testing (13) to determine the concentration-response curves, their slope and the EC<sub>50</sub> values with 95% confidence limits (16) (3). The NOEC may be determined by an appropriate statistical test outlined in (13), such as the Fisher's exact test (4).

29. Where the standard methods of calculating the EC<sub>50</sub> are not applicable to the data obtained, the highest concentration causing no immobility and the lowest concentration producing 100 percent immobility should be used as an approximation for the EC<sub>50</sub> (this being considered the geometric mean of these two concentrations).

### *Test report*

30. The test report should include the following:

Test substance:

- physical nature and relevant physical-chemical properties;
- chemical identification data, including purity.

Test species:

- source and species of *Chironomus*, supplier of source (if known) and the culture conditions used (including source, kind and amount of food, feeding frequency).

Test conditions:

- description of test vessels: type and volume of vessels, volume of solution, number of chironomids per test vessel, number of test vessels (replicates) per concentration;
- methods of preparation of stock and test solutions including the use and concentration of any solvent or dispersants, test concentrations used;
- details of dilution water: source and water quality characteristics (pH, hardness, alkalinity, conductivity, etc.); composition of reconstituted water if used;
- incubation conditions: temperature, light intensity and periodicity, dissolved oxygen, pH, etc.

Results:

- the number and percentage of chironomid larvae that were immobilised or showed any adverse effects (including abnormal behaviour) in the controls and in each treatment group, at each observation time, and a description of the nature of the effects observed;
- results and date of a test performed with a reference substance, if available;
- the nominal test concentrations and the result of all analyses to determine the concentration of the test substance in the test vessels; the recovery efficiency of the method and the limit of quantification (LoQ) should also be reported;

- all physical-chemical measurements of temperature, pH and dissolved oxygen made during the test;
- the  $EC_{50}$  at 48 h for immobilisation with 95% confidence intervals and a graph of the fitted model, the slope of the concentration-response curve and its standard error; statistical/mathematical procedures used for determination of the  $EC_{50}$  (these data items for immobilisation at 24 h should also be reported when they were measured).
- the NOEC at 24 h and at 48 h if applicable.
- explanation for any deviation from the TG and whether the deviation affected the test results.



## LITERATURE

- (1) Environment Canada (1995), Guidance document on measurement of toxicity test precision using control sediments spiked with a reference toxicant, Report EPS 1/RM/30.
- (2) Environment Canada (1997), Test for growth and survival in sediment using larvae of freshwater midges (*Chironomus tentans* or *Chironomus riparius*), Biological Test Method, Report EPS 1/RM/32.
- (3) Finney DJ (1978), Statistical methods in biological assay, 3<sup>rd</sup> ed., Griffin, Weycombe, London, UK.
- (4) Fisher RA (1922), On the interpretation of  $\chi^2$  from contingency tables, and the calculation of P. *J Royal Stat Soc* 85: 87-94.
- (5) Fleming R, Crane M, van de Guchte C, Grootelaar L, Smaal A, Ciarelli S, Karbe L, Borchert J, Westendorf J, Vahl H, Holwerda D, Looise B, Guerra M, Vale C, Castro O, Gaudencio MJ, van den Hurk P (1994), Sediment toxicity tests for poorly water-soluble substances, Final Report to the European Commission, Report No. EC 3738, WRc, UK.
- (6) Hill IR, Matthiessen P, Heimbach F, editors (1993), Guidance document on sediment toxicity tests and bioassays for freshwater and marine environments, Society of Environmental Toxicology and Chemistry-Europe, Brussels, Belgium.
- (7) Kawai K (1986), Fundamental studies on chironomid allergy, I. Culture methods of some Japanese chironomids (Chironomidae, Diptera), *Jp J Sanit Zool* 37: 47-57.
- (8) Milani D, Day KE, McLeay DJ, Kirby RS (1996), Recent intra- and inter-laboratory studies related to the development and standardisation of Environment Canada's biological test methods for measuring sediment toxicity using freshwater amphipods (*Hyalella azteca*) and midge larvae (*Chironomus riparius*), Technical Report, Environment Canada, National Water Research Institute, Burlington, Ontario, Canada.
- (9) OECD (2000), *Guidance document on aquatic toxicity testing of difficult substances and mixtures*, Series on Testing and Assessment No. 23, ENV/JM/MONO(2000)6, OECD, Paris.
- (10) OECD (2004a), *Sediment-water chironomid toxicity test using spiked sediment*, Test Guideline No. 218, OECD Guidelines for the Testing of Chemicals, OECD, Paris.
- (11) OECD (2004b), *Sediment-water chironomid toxicity test using spiked water*, Test Guideline No. 219, OECD Guidelines for the testing of chemicals, OECD, Paris.
- (12) OECD (2004c), *Daphnia sp., acute immobilisation test*, Test Guideline No. 202, OECD Guidelines for the testing of chemicals, OECD, Paris.
- (13) OECD (2006), *Current approaches in the statistical analysis of ecotoxicity data: a guidance to application*, OECD Series on Testing and Assessment, No. 54, ENV/JM/MONO(2006)18, OECD, Paris.
- (14) OECD (2010), *Sediment-Water Chironomid life-cycle toxicity test using spiked water or spiked sediment*. Test Guideline No. 233, OECD Guidelines for the Testing of Chemicals, OECD, Paris.

- (15) OECD (2011), Validation Report for the acute Chironomid Assay, Series on Testing and Assessment No.144, ENV/JM/MONO(2011)25, OECD, Paris
- (16) Stephan CE (1977), Methods for calculating an LC50, In: Aquatic toxicology and hazard evaluation (Mayer FI, Hamelink JL, editors), ASTM STP 634-American Society for Testing and Materials, p 65-84.
- (17) Streloke M, Köpp H, editors (1995), Long-term toxicity test with *Chironomus riparius*: Development and validation of a new test system. Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft, No. 315, BBA, Berlin, Germany.
- (18) [USEPA] U.S. Environmental Protection Agency (1996a), Whole sediment acute toxicity invertebrates, U.S. Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances, OPPTS 850.1735, Washington, DC, USA.
- (19) [USEPA] U.S. Environmental Protection Agency (1996b), Chironomid sediment toxicity test, U.S. Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances, OPPTS 850.1790, Washington, DC, USA.
- (20) [USEPA] U.S. Environmental Protection Agency (2000), Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates, 2<sup>nd</sup> edition, EPA 600/R-99/064, U.S. Environmental Protection Agency, Washington, DC, USA.
- (21) Weltje, L, Rufli, H, Heimbach, F, Wheeler, J, Vervliet-Scheebaum, M, Hamer, M. (2010), The chironomid acute toxicity test: development of a new test system, Integrated Environmental Assessment and Management 6: 301-307.

## ANNEX 1

## DEFINITIONS/DESCRIPTION

In the context of this TG, the following definitions/desriptions are used:

**EC<sub>50</sub>** is the concentration estimated to immobilise 50 per cent of the chironomid larvae within a stated exposure period.

**Immobilisation:** Those animals that are not able to change their position (by crawling or swimming movements) within 15 seconds after mechanical stimulation, e.g. by subjecting the larvae to a gentle stream of water from a Pasteur pipette or agitation of the test vessel, are considered to be immobilised. The movements of larvae are erratic and phases of high activity (i.e. swimming by quickly whipping their body into an alpha shape when most contracted and then inside out again) are interrupted by phases of no movement. Also, missing larvae are considered immobile.

## ANNEX 2

RECOMMENDATIONS FOR CULTURING *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). Also, filter paper has been used as substrate. 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). It is advisable to regularly exchange egg ropes with other laboratories to minimize inbreeding.
2. Cages should be held at room temperature or in a constant environment room at  $20 \pm 2^\circ\text{C}$  with a photo period of 16 hour light (intensity ca. 1000 lux), 8 hours dark. Including a half-hour dawn and/or dusk phase with lower light intensity of ca. 500 lux has been shown to stimulate swarming and reproduction (1). 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 should be aerated for ca. 24 h 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. This should be done at least once a month or when the water quality deteriorates.

***Feeding larvae***

4. *Chironomus* larvae should be fed with a fish flake food (TetraMin<sup>®</sup>, TetraPhyll<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 should 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. A cotton wool pad soaked in a saturated sucrose solution may serve as a food for emerged adults. This can enhance life-span and improve reproduction (4).

### *Emergence*

8. At  $20 \pm 2^\circ\text{C}$ , adults will begin to emerge from the larval rearing vessels after approximately 13 - 15 days. Males are easily distinguished by having plumose antennae and a thin body.

### *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 required for starting a toxicity test or setting up new culture vessels, the egg masses should be carefully removed. They should be transferred to a small dish containing a sample of the breeding water. Egg masses can be used directly to start a new culture vessel (e.g. 2 - 4 egg masses/vessel) or hatching is awaited and a number of defined larvae (e.g. 200) are introduced into a new culture vessel.

10. At  $20^\circ\text{C}$ , 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 (2) 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". The test solution should not be prepared according to Elendt and Bias (3) for the concentrations of  $\text{NaSiO}_3 \cdot 9\text{H}_2\text{O}$ ,  $\text{NaNO}_3$ ,  $\text{KH}_2\text{PO}_4$  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.

**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> · 4H <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> · 6H <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> · 2H <sub>2</sub> O <sup>(1)</sup>	1260	1.0	0.25	0.063	0.016
CuCl <sub>2</sub> · 2H <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
CaCl <sub>2</sub> · 6H <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 · 2H <sub>2</sub> O <sup>(1)(2)</sup>	5000	20.0	5.0	2.5	0.625
FeSO <sub>4</sub> · 7H <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> · 2H <sub>2</sub> O	293800	1.0	293.8
MgSO <sub>4</sub> · 7H <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> · 9H <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

- (1) Credland, P.F. (1973), A new method for establishing a permanent laboratory culture of *Chironomus riparius* Meigen (Diptera: Chironomidae). *Freshwater Biology* 3: 45-51.
- (2) Elendt, B.P. (1990), Selenium deficiency in Crustacea. *Protoplasma*, 154: 25-33.
- (3) Elendt, B.P. and W.-R. Bias (1990), Trace nutrient deficiency in *Daphnia magna* cultured in standard medium for toxicity testing. Effects on the optimization of culture conditions on life history parameters of *D. magna*. *Water Research*, 24: 1157-1167.
- (4) OECD (2010), *Validation report of the chironomid full life-cycle toxicity test*, Series on Testing and Assessment No. 136, ENV/JM/MONO(2010)35, OECD, Paris.

## ANNEX 3

**CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER**

Experience has shown that natural waters with the following characteristics (Table 4) are suitable as holding water and dilution water for chironomids.

**Table 4. 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
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 should not be used in this situation).



## ANNEX 4

**RESULTS OF THE RINGTEST WITH THE REFERENCE COMPOUNDS POTASSIUM-CHLORIDE AND 3,5-DICHLOROPHENOL**

The 24-h and 48-h EC<sub>50</sub> values for the reference compounds potassium-chloride (KCl) and 3,5-dichlorophenol (3,5-DCP) that were obtained in the ring test supporting this TG, are summarized below. The full data are presented in the validation report (ref.15 in the TG). The valid EC<sub>50</sub> values are used to derive approximate ranges, to which results of reference tests (see paragraph 5) can be compared with.

Statistical analysis of the EC<sub>50</sub> values was conducted with the software programme GraphPad Prism version 5.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

	3,5-DCP 24-h	3,5-DCP 48-h	KCl 24-h	KCl 48-h
Number of values	13	13	15	15
Minimum	0.770	0.517	1.53	0.330
25% Percentile	1.68	0.776	2.16	1.10
Median	2.30	1.67	2.84	1.44
75% Percentile	3.47	2.15	3.80	1.85
Maximum	5.74	3.86	5.94	2.19
10% Percentile	<b>0.807</b>	<b>0.541</b>	<b>1.54</b>	<b>0.643</b>
90% Percentile	<b>5.21</b>	<b>3.57</b>	<b>5.34</b>	<b>2.09</b>
Coefficient of variation	53.89%	60.12%	39.97%	35.92%

Based on the 10% and 90% percentiles, the reference ranges (rounded values) could be estimated as follows:

3,5-DCP:   **24-h EC<sub>50</sub>**   **0.80 – 5.3 mg/L**       **48-h EC<sub>50</sub>**   **0.50 – 3.6 mg/L**

KCl:       **24-h EC<sub>50</sub>**   **1.5 – 5.4 g/L**       **48-h EC<sub>50</sub>**   **0.60 – 2.1 g/L**

These ranges should be considered as guidance values to study the sensitivity of the animals from the test culture over time, especially when significant changes in culturing conditions are made or when new organisms are added to the culture (see paragraph 5).

The KCl values are a bit more homogeneous than those of 3,5-DCP (as evidenced by their coefficients of variation). Some characteristics of KCl, i.e. the high water solubility, no degradation and the ca. 1000-fold higher test concentrations required to invoke 50% effect, have likely contributed to reduce experimental error in the laboratory.