Section 2
Effects on Biotic Systems

Test Guideline No. 253

Short-term juvenile hormone (JH) activity screening assay in Daphnia magna

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OECD Guidelines for the Testing of Chemicals

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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Short-term Juvenile Hormone Activity Screening Assay using Daphnia magna (JHASA)

INTRODUCTION

1. This Test Guideline (TG) describes a short-term juvenile hormone (JH) activity screening assay using *Daphnia magna* to detect the potential of chemicals with JH activity. The JHASA was designed as a screen assay which evaluates male offspring production in the parthenogenetic daphnid, as described in Annex 7 of OECD TG 211 (*Daphnia magna* Reproduction Test). The JHASA is placed at level 3 of the OECD Conceptual Framework for the testing of endocrine disrupting chemicals (EDCs) in Guidance Document 150 (1) for mechanistic information and is not intended to determine toxicity values (i.e. no observed effect concentration [NOEC] or Effect concentration for x% effect [ECx]) for risk assessment, unless there is a concentration response curve based on more than three treatment levels.

2. The concept of the JHASA is based on the studies of male offspring production in the parthenogenetic daphnids, which is regulated by JH signaling pathway (2, 3). Production of male offspring is usually known to occur under environmental stimuli, such as shortening of photoperiod, lowering temperature, decreasing quantity of food, and increasing population density (4-6). However, male offspring production is also induced by the exposure to innate JHs of crustaceans and insects (methyl farnesoate for crustacean and JH I, II, III for insects) and JH analogs (JHAs) such as insect growth regulators (e.g. hydroprene, pyriproxyfen, fenoxycarb) (7-10) and could lead to population-relevant effects (11). To use this male offspring production as an additional mean of detecting chemicals with JH activity, inter-laboratory validation study was conducted (12) and evaluation of male offspring production was added to Annex 7 of TG 211 *Daphnia magna* Reproduction Test as an optional measurement endpoint in 2008 (13).

3. TG 211 Annex 7 can provide sufficient data for hazard and risk assessment; however, this test requires additional resources and cost to identify sex of all offspring produced for 21 days. Thus, the Short-term Juvenile Hormone Activity Screening Assay using *Daphnia magna* (JHASA), was developed to shorten the test period from 21 days in TG211 Annex 7 to approximately 7 days in JHASA (14-16). Based on the knowledge that sex determination of offspring occurs during the critical period of oocyst development in ovary, approximately 7-10 h before ovulation to the brood chamber (17), JHASA starts exposure from mature adult females (e.g. 10-17 days old) and only observes offspring from the second brood after the exposure. The first brood in the brood chamber at the start of the test is not included in the observation because its sex has been already determined prior to the exposure.

PRINCIPLE OF THE TEST

4. The mature females (parent animal) with developing embryos in their brood chambers are exposed to at least three concentrations of test chemical and a water control (and a solvent control if necessary). The test duration is approximately 7 days, until the test daphnids reproduce the second brood after the exposure. Test duration may be extended until all surviving daphnids produce the second brood (see section "*Duration*" for more detail). Ten vessels or replicates (each vessel containing 1 mature female) are used for each treatment. The number of female and male offspring of the second brood is counted. Identification of offspring sex is performed by macroscopic observation of the first antenna, which is longer in males than in females. Mortality of parent animals is also recorded. The proportion of male offspring (male ratio) is calculated in each of treatment replicates when the second brood is produced. The summary of the test parameters and conditions can be found in Annex 2.

5. The JHASA serves as a screen for chemicals having JH-like activity which can be detected by production of male offspring in the test concentrations. If no male offspring is observed in control(s), the test chemical having male offspring induction may be considered 'active' without statistical analysis. However, if a few males are found in control(s) or if only a few males are induced in the exposures, male ratio should be analyzed to determine statistically significant differences between the exposures and control(s) response (see Annex 6 for more details). In addition, if the possibility that males are induced by other environmental factors (e.g. feeding, density, photoperiod, temperature) cannot be completely excluded, it is recommended to check the reproducibility of the test and to consider whether other factors may have influenced the results. These consideration will inform whether further testing (i.e. TG 211 including sex ratio determination as described in Annex 7) is required for the test chemical to determine the toxicity values (i.e. NOEC or ECx) (see (1) for more details).

INFORMATION ON THE TEST CHEMICAL

6. The water solubility and the vapour pressure of the test chemical should be known. Other useful information includes the structural formula, purity of the test chemical, stability in water and light, pKa, Pow and biodegradability (e.g., by TG301 or 310) (18, 19). Guidance for testing chemicals with physical chemical properties that make them difficult to test is provided in the OECD guidance document No. 23 (20). A reliable analytical method for the quantification of the chemical in the test solutions with reported recovery efficiency and limit of determination (LOD) and quantification (LOQ) should be available, if analytical verification of exposure is required.

7. Although it is not mandatory, results of an acute immobilisation toxicity test (e.g by TG 202) (21) performed with the same strain of *D. magn*a may provide useful information in selecting an appropriate range of test concentrations in the reproduction tests. Results of *in vitro* assays, such as the JH receptor transcriptional activation assay, might also be useful (22, 23) to confirm results.

REFERENCE SUBSTANCES

8. A reference substance may be tested regularly as a means of assuring that the test conditions are reliable. A JH agonist, diofenolan, which was used in international ring-tests (24) is recommended for this purpose. Other JH agonists used successfully in JHASA are JH III, methyl farnesoate, fenoxycarb, pyriproxyfen. (14)(15)(16). Test(s) with a reference substance should be conducted as necessary, for example after introducing new strain organisms or significant changes in culture conditions. However, the tests are recommended to be conducted at least twice a year.

VALIDITY OF THE TEST

9. For a test to be valid, the following criteria should be met in the control(s):
   - The mortality of the parent animals (female *Daphnia*) should not exceed 20% at the end of the test;
   - The mean number of living offspring produced per alive parent animal should be ≥12;
   - No more than one parent animal should produce male offspring;
   - The mean male ratio should not exceed 5%.

10. The last three criteria above were established to ensure the statistical detection power on male ratio (24). If a deviation from the test validity criteria is observed, the consequences of the deviation(s) in relation to the reliability of the test results should be considered and these considerations should be included in the report.

DESCRIPTION OF THE METHOD

Apparatus

11. Test vessels and other apparatus, which will come into contact with the test solutions, should be made entirely of glass or other chemically inert material. The test vessels will normally be glass beakers.

12. In addition some or all of the following equipment will be required:
   - oxygen meter (with microelectrode or other suitable equipment for measuring dissolved oxygen in low volume samples);
   - adequate apparatus for temperature control;
   - pH-meter;
   - equipment for the determination of the hardness of water;
   - adequate apparatus for the control of the lighting regime and measurement of light intensity.

Test Organism

13. The species to be used in the test is *D. magna* Straus. Other daphnids (e.g., *Ceriodaphnia dubia* (25)) may be used provided they meet the validity criteria as appropriate (the validity criterion relating to the reproductive output in the controls should be relevant for all species).

If other daphnids are used, they should be clearly identified and rationale for their use should be reported together with any adaptations to the Test Guideline's recommendations.

14. Preferably, the *Daphnia* clone should have been identified by genotyping. Considering the purpose of the test, strains that are less likely to produce male offspring in control condition are suitable. For example, the mean male ratio of the test strain should not exceed 5% in control condition to meet performance criteria. The ring test has shown that strain from NIES (National Institute for Environmental Studies) and USEPA (United States Environmental Protection Agency), Clone A strain (The strain named as "Clone A" in the previous ring test for TG 211, which originated from IRCHA in France) (13, 26), and DHI strain (DHI Water & Environment, Denmark) are acceptable, which did not produce male offspring in the controls in the ring test for JHASA (24). However, Clone A sometimes produces males in controls (24), so care should be taken to maintain healthy stock without males by adjusting optimal density and feeding condition.

15. At the start of the test, female *Daphnia* aged 10-17 days old are recommended to be used. *Daphnia* outside this age range may be used, however, to obtain a sufficient number of offspring produced in the test, young females that has not yet produced the first brood should be avoided. When using *Daphnia* older than 3-week-old, prior confirmation is recommended to ensure that parent mortality in the control(s) does not exceed 20% in the test. They should be derived from a healthy stock (i.e. showing no signs of stress such as high mortality, presence of males and ephippia, delay in the production of the first brood, discoloured animals, etc) and should not be first brood progeny. The stock animals should be maintained in culture conditions (light, temperature, medium, feeding and animals per unit volume) similar to those to be used in the test. If the medium used in the test is different from that used for routine culture, it is good practice to include a pre-test acclimation period of normally about 3 weeks (i.e. one generation) to avoid stressing the parent animals.

Test medium

16. It is recommended that a fully defined medium be used in this test. This can avoid the use of additives (e.g. seaweed, soil extract), which are difficult to characterise, and, therefore, improves the opportunities for standardisation between laboratories. Elendt M4 (27) and M7 media (see Annex 3) have been found to be suitable for this purpose. However, other media (e.g. 28, 29) are acceptable provided the performance of the *Daphnia* culture is shown to meet the validity criteria for the test.

17. If media are used which include undefined additives, these additives should be specified clearly and information should be provided in the test report on composition, particularly with regard to carbon content as this may contribute to the diet provided. It is recommended that the total organic carbon (TOC) and/or chemical oxygen demand (COD) of the stock preparation of organic additive be determined and an estimate of the resulting contribution to the TOC/COD in the test medium made. It is further recommended that TOC levels in the medium (i.e. before addition of the algae) be below 2 mg/l (30).

18. When test chemicals containing metals, it is important to recognise that the properties of the test medium (e.g. hardness, chelating capacity) may have a bearing on the availability and toxicity of the test chemical. For this reason, a fully defined medium is desirable.

However, at present, the only fully defined media which are known to be suitable for long-term culture of *D. magna* are Elendt M4 and M7. Both media contain the chelating agent EDTA. Work has shown (26) that the 'apparent toxicity' of cadmium is generally lower when the reproduction test is performed in M4 and M7 media than in media containing no EDTA. M4 and M7 are not, therefore, recommended for testing chemicals containing metals, and other media containing known chelating agents should also be avoided. For metal-containing chemicals it may be advisable to use an alternative medium such as, for example, ASTM reconstituted hard fresh water (30), which contains no EDTA. This combination of ASTM reconstituted hard fresh water and seaweed extract (31) is suitable for long-term culturing of *D. magna* (26).

19. The dissolved oxygen concentration should be above 3 mg/L at the beginning and during the test. The pH should be within the range 6-9, and normally it should not vary by more than 1.5 units in any one test. Hardness above 140 mg/L (as $CaCO_3$) is recommended. Tests at this level and above have demonstrated reproductive performance in compliance with the validity criteria (32, 33).

Test solutions

20. Test solutions of the chosen concentrations are usually prepared by dilution of a stock solution. The stock solutions should preferably be prepared by simply mixing or agitating the test chemical in test medium using mechanical means (e.g. stirring and/or ultra-sonication). If the test chemical is not stable under test condition and/or difficult to dissolve in water, procedures described in the Guidance Document No. 23 should be followed (20). 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 stock solution. Where a solvent cannot be avoided, Guidance Document No. 23 (20) should be consulted. Only low toxicity solvent (i.e. acetone, ethanol, methanol, tertiary-butyl alcohol, acetonitrile, dimethylformamide, dimethyl sulfoxide, and triethylene glycol) (20) should be used whilst solvent of unknown toxicity should not be used. The final concentration of the solvent used should be minimised as far as possible (not exceeding 100 mg/L or 0.1 mL/L) and should be the same in all test vessels, excluding the dilution water control (20). When a solvent is used, an additional solvent control is required in addition to the dilution water control.

21. The test should generally be conducted without the adjustment of pH. If the pH does not remain in the range 6-9, then a second test could be carried out, adjusting the pH of the stock solution to that of the dilution water before preparing test the test solutions. The pH adjustment, preferably with HCl and 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 chemical, is caused.

PROCEDURE

Conditions of Exposure

Duration

22. The exposure can be terminated as soon as the second brood is produced in each vessel. Because the reproduction cycle of *D. magna* is typically 2-3 days, the second brood production usually occurs within 7 days. If delayed reproduction occurs in the exposure groups, test duration may be extended until all surviving daphnids produce the second brood. Given the properties as a screening assay and that reproduction may also be inhibited in the higher concentrations, it is recommended to end the test within 10 days, regardless of the number of test concentrations and/or replicates producing the second brood. The third brood, if produced, is not included in the data evaluation.

Loading

23. Parent animals are allocated individually, one per test vessel, usually with 50 -100 mL for *D. magna* of medium in each vessel, unless a flow-through test design is necessary for testing. The flow-through system may be required when a test chemical degrades very rapidly (see also section “Test medium renewal”).

24. Larger volumes may sometimes be necessary to meet requirements of the analytical procedure used for determination of the test chemical concentration, although pooling of replicates for chemical analysis is also allowable. If volumes greater than 100 mL are used, the ration given to the *Daphnia* may need to be increased to ensure adequate food availability and compliance with the validity criteria (see the section Feeding).

Preparation of Test animals

25. At least 10 animals are individually held at each test concentration and control(s).

26. Use adult females who have eggs in the brood chamber. The susceptible period of sex determination is the late oocytes developmental stage in ovary, which is 7-10 h before releasing of the previous brood in the brood chamber (15, 17). When neonates from the first brood have hatched in the brood chamber, it is possible that the sex of the neonates from the second brood in the ovary has already been determined (Figure 1). Therefore, the daphnids with hatched neonates in the brood chamber (>48 h after ovulation) should not be used because the susceptible period of sex determination of the second brood may be missed.

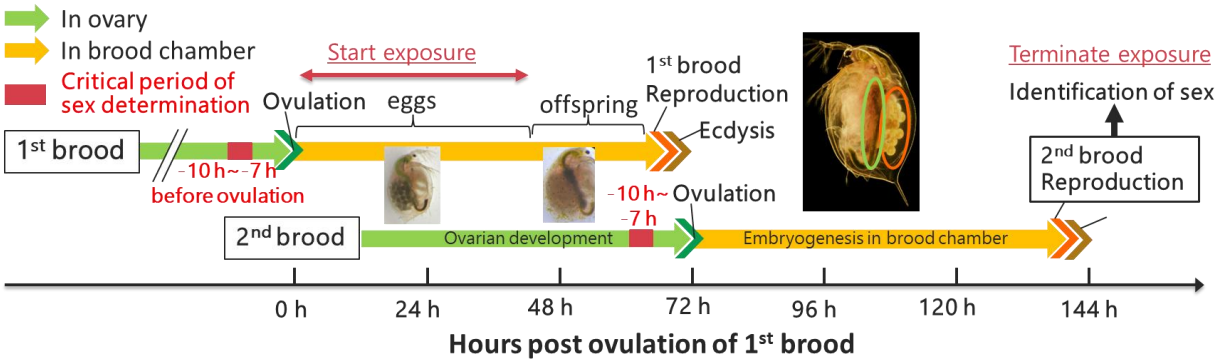

Figure 1. Reproduction cycle and the critical period of sex determination of 1st and 2nd brood during JHASA

27. Treatments should be allocated to the test vessels and all subsequent handling of the test vessels should be done in a random procedure. Failure to do this may result in bias that could be construed as being a concentration effect. In particular, if experimental units are handled in treatment or concentration order, then some time-related effect, such as operator fatigue or other error, could lead to greater effects at the higher concentrations.

Test concentrations

28. The purpose of this assay is to determine whether a test chemical has potential JH activity; therefore, it is recommended to use as high a concentration as possible to the extent that the parent animals do not die and have reproduction. For example, a half concentration of the EC50 from acute immobilisation test may be used as the highest concentration. Alternatively, the maximum test concentration should be set by the solubility limit of the test chemical in the test medium, the maximum tolerated concentration (MTC) (the highest test concentration of the chemical that results in ≤20% mortality), or a maximum concentration of 100 mg/L, whichever is the lowest.

29. Normally, there should be at least three test concentrations and arranged in a geometric series with a separation factor preferably not exceeding 3.2. Justification should be provided if fewer than three concentrations are used. Care should be taken not to have a series of test concentrations where no offspring are observed due to parent mortality. Additional consideration to ensure ≤20% mortality in the lower concentrations may be required by some regulatory authorities.

30. Considering the sensitivity difference of each strain in each laboratory condition (24, 34), the laboratory is recommended to conduct a range-finding test or acute immobilization test beforehand in order to set the highest concentration where parent daphnids can survive during the test. Three concentrations from the half of EC50 have been successfully used for most chemicals without >20% parent mortality; however, it is recommended to conduct a range finding test for certain chemicals (e.g. slow-acting toxic chemicals). The range-finding test is conducted with, for example, five replicates for each treatment and control. Additional information, from tests with similar compounds or from literature, on acute and chronic toxicity to *Daphnia* and/or other aquatic organisms may also be useful in deciding

on the range of concentrations to be used in the range-finding test. In addition, the second brood production needs to be ensured within approximately 7 days (maximum 10 days) to observe the male ratio of the second brood offspring.

31. If no effects are observed at the highest concentration in the range-finding test (e.g. at 100 mg/L), or when the test chemical is likely to be low/no toxicity based on lack of toxicity to other organisms and/or low/no uptake, the JHASA may be performed as a limit test, using a limit test concentration (e.g.100 mg/L) and the control. Ten replicates should be used for both the treatment and the control groups. A limit test will provide the opportunity to demonstrate that there is no statistically significant effect at the limit concentration, but if male offspring is recorded at the limit concentration, a full test will normally be required.

Feeding

32. For semi-static tests, feeding should preferably be done daily, but at least three times per week (i.e. corresponding to media changes). If the test starts on Friday and feeding is not provided on weekends, twice volume of food should be fed at the test initiation. The possible dilution of the exposure concentrations by food addition should be taken into account and avoided as much as possible by using appropriate concentration of algae suspensions. A sufficiently concentrated algal suspension should be fed to the *Daphnia* to minimise the volume of algal culture medium transferred to the test vessels. Concentration of the algae can be achieved by centrifugation followed by re-suspension in *Daphnia* culture medium. Deviations from this (e.g. for flow-through tests) should be reported.

33. During the test, the diet of the parent animals should preferably be living algal cells of one or more of the following: *Chlorella* sp, *Raphidocelis subcapitata* (formerly *Pseudokirchneriella subcapitata*) and *Desmodesmus subspicatus* (formerly *Scenedesmus subspicatus*). The supplied diet should be based on the amount of organic carbon (C) provided to each parent animal. Research (35) has shown that, for *D. magna*, ration levels of between 0.1 and 0.2 mg C*/Daphnia*/day are sufficient for achieving the required number of living offspring to meet the test validity criteria for TG211 (13). The ration can be supplied at a constant rate throughout the period of the test. Food limitation is one of the environmental factors which induce male production (4-6); however, this ration was confirmed to be sufficient to prevent male production (12)(24).

34. If surrogate measures, such as algal cell number or light absorbance, are to be used to feed the required ration level (i.e. for convenience since measurement of carbon content is time consuming), each laboratory should produce its own nomograph relating the surrogate measure to carbon content of the algal culture (see Annex 4 for advice on nomograph production). Nomographs should be checked at least annually and more frequently if algal culture conditions have changed. Light absorbance has been found to be a better surrogate for carbon content than cell number (36).

35. In addition, yeast, cerophyll and trout chow (YCT) could be provided (e.g. 50 µL/daphnid in 50 mL/day) to support sufficient reproduction.

Light

36. 16 h light at an intensity not exceeding 15-20 $\mu E \cdot m^{-2} \cdot s^{-1}$ measured at the water surface of the vessel. For light-measuring instruments calibrated in lux, an equivalent range of 1000-1500 lux for cool white light corresponds close to the recommended light intensity 15-20 $\mu E \cdot m^{-2} \cdot s^{-1}$. Constant darkness conditions should not be used because slight increased male offspring in culturing condition was reported in NIES and Clone A strain (24).

Temperature

37. The temperature of the test media should be within the range 18-22°C. However, for any one test, the temperature should not, if possible, vary by more than 2°C within these limits (e.g. 18-20, 19- 21 or 20-22°C) as daily range. It may be appropriate to use an additional test vessel for the purposes of temperature monitoring.

Aeration

38. The test vessels should not be aerated during the test.

Test medium renewal

39. The frequency of medium renewal will depend on the stability of the test chemical, but should be at least three times per week. For a readily degradable chemical, frequent renewal (e.g. everyday) is recommended until ovulation of the second brood egg (or releasing of the first brood offspring) to secure the exposure of chemicals during its sex determination period (i.e. 7-10 h before ovulation to the brood chamber) (15, 17).

40. When the medium is renewed in semi-static tests, a second series of test vessels are prepared and the parent animals transferred to them by, for example, a glass pipette of suitable diameter. The volume of medium transferred with the *Daphnia* should be minimised. If the flow-through system with continued flow of test solutions is used, minimum of four volume exchanges per day may be required (37)

Observations

Offspring

41. The first brood offspring produced by each parent animal should preferably be recorded (but counting is not mandatory) and removed daily to prevent them consuming food intended for the parent. The second brood offspring should be counted and subjected to identification of sex. For the purpose of this guideline, it is only the number of living offspring from the second brood that needs to be counted, but the presence of aborted eggs or dead offspring is preferably recorded.

Identification of offspring sex

42. After the exposure start, male ratio of the living offspring from the second brood is confirmed. The most practical and easy way to differentiate sex of *Daphnia* is to use their phenotypic characteristics. Males and females are different in the length and morphology of the first antennae, which are longer in males than females (Fig. 2). This difference is recognizable right after birth, although other secondary sex characteristics develop as they grow up (38). To observe the morphological sex, neonates produced by each test animal should be transferred by pipet and placed into a petri dish with test medium. The medium is kept to a minimum to restrain movement of the animals. Observation of the first antennae can be conducted under a stereomicroscope (×10-60). Dead offspring is excluded from the observation because the sex identification sometimes difficult due to the loss of first antenna after death.

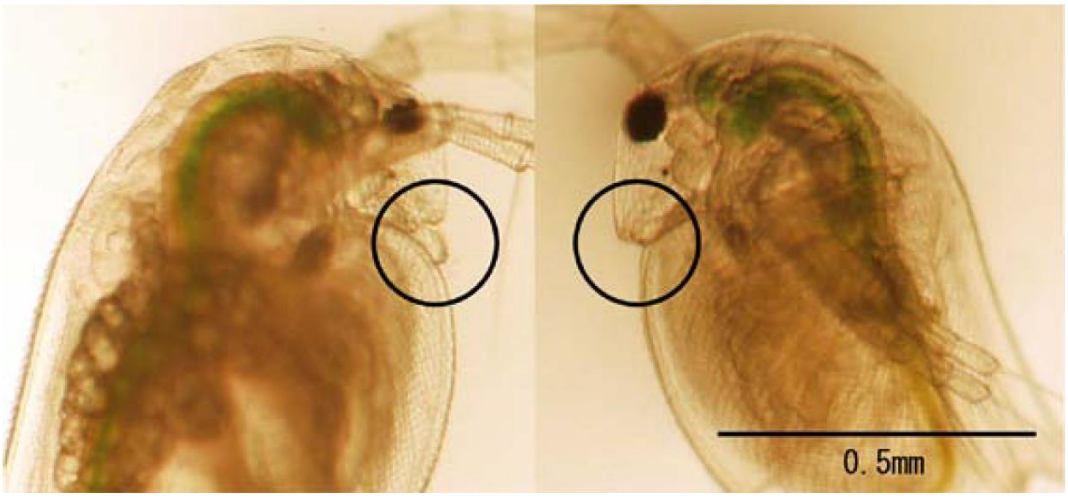

Figure 2. 24-hour-old male (left) and female (right) of *D. magna*.

Males (left) can be distinguished from females (right) by the length and morphology of the first antennae as shown in the circles (39).

Parent mortality

43. Mortality among the parent animals should be recorded preferably daily, or at least as frequently as offspring are counted.

Other parameters

44. The ecdysis of each parent animal may also be useful to distinguish the second brood from the first brood.

Analytical measurements

Concentration of the test chemical

45. During the test, the concentrations of test chemical in solution are determined as a minimum, in the control(s), the highest and lowest test concentrations, but preferably in all treatments. In static tests, analyses should be made at the beginning and the end of the test. In semi-static tests, analyses should be made when freshly prepared and at the time of renewal on at least one occasion during the test (i.e. a sample from the same solution - when freshly prepared, 'new sample', and at renewal, 'old sample'). If a flow-through test is used, it is recommended that the actual test chemical concentrations are measured twice, including at the start of the test in one vessel from each treatment as a minimum.

46. It is recommended that the results are based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test chemical 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 initial values. If the deviation from the nominal or measured initial concentration is greater than ± 20%, results should be expressed in terms of the geometric mean or the time-weighted mean; see Annex 5 for more details.

Physical-chemical parameters

47. The dissolved oxygen concentration, temperature, and pH values should be measured at least one renewal period, in fresh and old media, in the control(s) and in the highest test chemical concentration. The dissolved oxygen concentration should be ≥3 mg/L in the control(s) and test vessels at the beginning and during the test. The temperature is preferably recorded continuously throughout the test or, as minimum, at the beginning and the end of the test, and should not, if possible, vary by more than 2°C during the test. The pH should be within the range of 6-9. Hardness is recommended to be measured in the control(s) and one test vessel at the highest concentration at the start and the end of the test.

DATA AND REPORTING

Treatment of results

48. The most important endpoint of this test is male ratio in the second brood offspring. The male ratio (the number of male offspring/the total number of offspring) in the second brood should be calculated for each parent animal (i.e. replicate) and average male ratio be calculated per test condition (test concentrations and control(s)). The average of the number of female and male offspring per parent animal can be calculated for each test condition, respectively. In addition to the ecdysis of the pro-individual, the presence of aborted egg and stillbirth of the offspring need to be reported, when observed during the test.

49. If the second brood production is not available in the vessels due to the parent mortality or due to adverse effects on the reproduction, including aborted egg or dead offspring, those replicates (vessels) should be excluded from the analysis of the male ratio in the second brood.

50. The total number of living offspring per parent animal can be calculated based on the production of living offspring by the surviving parent animals. However, the ecologically most relevant response variable is the total number of living offspring produced per parent animal which does not die accidentally or inadvertently during the test. If the parent animal dies accidentally or inadvertently during the test, then the replicate is excluded from the analysis. The analysis will then be based on a reduced number of replicates. If parental mortality occurs in exposed replicates, it should be considered whether or not the mortality follows a concentration-response pattern, e.g. if there is a significant regression of the response versus concentration of the test chemical with a positive slope (a statistical test like the Cochran-Armitage trend test may be used for this). If the mortality does not follow a concentration-response pattern, then those replicates with parental mortality should be excluded from the analysis of the test result. If the mortality follows a concentration-response pattern, the parental mortality should be assigned as an effect of the test chemical and the replicates should not be excluded from the analysis of the test result. The same validity criterion (20%) can be used for accidental and inadvertent parental mortality for the control(s) as well as for each of the test concentrations.

51. The number of surviving parents in the untreated control(s) is a validity criterion and should be documented and reported. Also, all other detrimental effects (e.g. abnormal behaviour) should be reported in the final report as well.

52. To identify potential JH activity of test chemical, the male ratio is compared between treatments and control groups. If NOEC/LOEC for the male ratio are required to express the effects, it should be analysed statistically as described in Annex 6.

Test report

53. The test report should include the following information:

Test chemical:

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

Test species:

- the strain (whether it has been genetically typed clone), supplier or source (if known) and the culture conditions used. If a different species to *D. magna* is used, this should be reported and justified.

Test conditions:

- test procedure used (e.g. semi-static or flow-through, volume,);
- photoperiod and light intensity;
- test design (e.g. number of replicates);
- details of culture medium used;
- detailed information on feeding, including amount (in mg C/daphnia/day) and schedule (e.g. type of food(s), including, for algae the specific name (species) and, if known, the strain, the culture conditions);
- method of preparation of stock solutions and frequency of renewal (the solvent or dispersant and its concentration should be given, when used).

Results:

- results from any preliminary studies on the stability of the test chemical;
- results from acute immobilisation test if available;
- the nominal test concentrations and the results of all analyses to determine the concentration of the test chemical in the test vessels; the recovery efficiency of the method and the limit of determination should also be reported;
- water quality within the test vessels (i.e. pH, temperature and dissolved oxygen concentration, and TOC and/or COD and hardness where applicable);
- the full record of the production of living offspring and its sex from the second brood during the test by each parent animal; time to production of offspring from the second brood; presence of aborted egg and dead offspring;
- the number of deaths among the parent animals and the day on which they occurred;
- plot of sex ratio of living offspring produced per parent animal in each replicate excluding any parent animal which may have accidentally or inadvertently died during the test vs. concentration of the test chemical;
- average of the number of offspring of the second brood and the male ratio in controls (control and solvent control) and exposed group are recorded;
- NOEC and LOEC for male ratio (if required); statistical analysis methods and treatment of data used;

- explanation for any deviation from the TG and whether the deviation affected the test results.

LITERATURE

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ANNEX 1: DEFINITIONS

For the purposes of this Guideline the following definitions are used:

Parent Animals are those female *Daphnia* present at the start of the test.

Offspring are the young *Daphnia* produced by the parent animals in the course of the test.

Brood: a group of offspring hatched in a brood chamber and released from the brood chamber at one time.

Male ratio: the number of living male offspring/the total number of living offspring in the brood

Ecdysis is molting, the periodic shedding of exoskeleton (carapace) by *Daphnia*

Accidental mortality: non chemical related mortality caused by an accidental incidence (i.e. known cause)

Inadvertent mortality: non chemical related mortality with no known cause

Mortality: An animal is recorded as dead when it is immobile, i.e. when it is not able to swim, or if there is no observed movement of appendages or postabdomen, within 15 seconds after gentle agitation of the test container. (If another definition is used, this should be reported together with its reference).

ECx is the concentration of the test chemical dissolved in water that results in a x% reduction in reproduction of *Daphnia* within a stated exposure period.

Lowest observed effect concentration (LOEC) is the lowest tested concentration of a test chemical at which the chemical is observed to have a statistically significant effect (at $p < 0.05$) when compared with the control. However, all test concentrations above the LOEC should have a harmful effect equal to or greater than those observed at the LOEC. When these two conditions cannot be satisfied, a full explanation should be given for how the LOEC (and hence the NOEC) has been selected.

No Observed Effect Concentration (NOEC) is the test concentration immediately below the LOEC, which when compared with the control, has no statistically significant effect ($p < 0.05$), within a stated exposure period.

ANNEX 2: TEST CONDITIONS FOR SHORT-TERM JUVENILE HORMONE ACTIVITY SCREENING ASSAY （JHASA）

1. Test species (recommended)	*Daphnia magna*
2. Test type	Semi-Static test, flow-through
3. Water temperature	20 ± 2 °C (e.g. 18-20, 19-21 or 20-22°C)
4. Illumination quality	Fluorescent bulbs (wide spectrum) 15-20 µE/m^{-2}/s^{-1}, 1000-1500 lux
5. Photoperiod	16 hours light, 8 hours dark
6. Test chamber size	50-100 mL
7. Age of test organisms at initiation	10-17 days
8. Number of organisms per replicate	1 daphnia/replicate vessel
9. Number of treatments	3 test chemical treatments at least plus appropriate control(s)
10. Number of replicates per treatment	10 replicates per treatment for test chemical and control (minimum)
11. Number of organisms per test	Minimum 40 *Daphnia* (minimum 50 *Daphnia*, if solvent control is used)
12. Feeding regime	Algal suspension (recommended rage of 0.1 – 0.2 mg C/*Daphnia*/day) Additional feeding of 0.05 mL YCT (Yeast, Cerophylle, and Trout Chow) per test vessel daily is recommended. If the test start from Friday and no feeding is provided on weekends, feed twice volume of each at the test initiation.
13. Aeration	None (The dissolved oxygen concentration should be above 3 mg/l at the beginning and during the test.)
14. pH	Within the range 6-9

15.	Dilution water	reconstituted hard fresh water (e.g., Elendt M4 and M7 media, Annex 3)
16.	Chemical Exposure duration	7 days (pre-exposure period is not required) It can be shortened or extended until all surviving *Daphnia* have second broods.
17.	Biological endpoints	Proportion of living male offspring in the second brood The number of living offspring (female, male) in the second brood (optional)
18.	Test validity criteria	The mortality of parent animal should not exceed 20% in control(s); The mean number of living offspring produced per alive parent animal should be ≥12 in control(s); No more than one parent animal should produce male offspring in control(s); The mean male ratio should not exceed 5% in control(s).

ANNEX 3: PREPARATION OF FULLY DEFINED ELENDT M7 AND M4 MEDIA

Acclimation to Elendt M7 and M4 media

Some laboratories have experienced difficulty in directly transferring *Daphnia* to M4 (40) and M7 media. However, some success has been achieved with gradual acclimation, i.e. moving from own medium to 30% Elendt, then to 60% Elendt and then to 100% Elendt. The acclimation periods may need to be as long as one month.

Preparation of stock solutions

The first publication of the M4 medium can be found in Elendt, B.P. (27). 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. Separate stock solutions (I) of individual trace elements are first prepared in water of suitable purity, e.g. deionised, distilled or reverse osmosis. A combined stock solution (II) (50-fold of the final concentration) is prepared from these stock solutions (I) (see Table 1). Each macro nutrient stock solution is prepared by adding each macro nutrient to 1 L of water as indicated in Table 2. A vitamin stock solution is prepared by adding three vitamins together to1 L of water as indicated in Table 3. The combined vitamin stock is stored frozen in small aliquots.

Preparation of M4 and M7 media

M4 and M7 media are prepared using the combined stock solution II, the macro nutrient stock solutions, and the combined vitamins stock solution. Fifty mL of the combined stock solution II, the amount of each macro nutrient stock solution which are given in Table 2, and 0.1 mL of the combined vitamin stock solution are made up to 1 L of water to prepare M4 and M7 media. To avoid precipitation of salts when preparing the complete media, add the aliquots of stock solutions to about 500-800 mL deionized water and then fill it up to 1 L. Vitamins are added to the media shortly before use. The medium is aerated and stabilised.

Table 1. Stock solution of trace elements for medium M4 and M7

Stock solutions (I)	Amount (mg) made up to 1 L of water (mg/L)	Concentration (related to medium M4)	To prepare the combined stock-solution II: mix the following amounts (mL) of stock solution (I) and make up to 1 L of water (mL/L)		Final concentrations in test solutions (mg/L)	
			M4	M7	M4	M7
H_3BO_3 (1)	57 190	20 000-fold	1.0	0.25	2.86	0.715
$MnCl_2$•4 H_2O (1)	7 210	20 000-fold	1.0	0.25	0.361	0.090
LiCl (1)	6 120	20 000-fold	1.0	0.25	0.306	0.077
RbCl (1)	1 420	20 000-fold	1.0	0.25	0.071	0.018
$SrCl_2$•6 H_2O (1)	3 040	20 000-fold	1.0	0.25	0.152	0.038
NaBr (1)	320	20 000-fold	1.0	0.25	0.016	0.004
Mo $Na2O_4$ •2 H_2O (1)	1 260	20 000-fold	1.0	0.25	0.063	0.016
$CuCl_2$•2 H_2O (1)	335	20 000-fold	1.0	0.25	0.017	0.004
$ZnCl_2$	260	20 000-fold	1.0	1.0	0.013	0.013
$CoCl_2$•6 H_2O	200	20 000-fold	1.0	1.0	0.010	0.010
KI	65	20 000-fold	1.0	1.0	0.0033	0.0033
Na_2SeO_3	43.8	20 000-fold	1.0	1.0	0.0022	0.0022
NH_4VO_3	11.5	20 000-fold	1.0	1.0	0.00058	0.00058
Na_2EDTA•2 H_2O (1)(2)	5 000	2 000-fold	20.0 (2)	5.0 (2)	2.5	0.625
$FeSO_4$•7 H_2O (1)(2)	1 991	2 000-fold			1.0	0.249

(1) The concentrations of these substances differ in M4 and M7, as indicated above.

(2) Na_2EDTA and $FeSO_4$ solutions are prepared individually, then poured together and autoclaved immediately. This Fe-EDTA solution is added to the stock solution II as indicated above.

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

Macro nutrient	Amount (mg) made up to 1 L of water (mg/L)	Concentration (related to medium M4 and M7)	Amount of macro nutrient stock solutions added to prepare M4 and M7 (mL/L)	Final concentrations in test solutions M4 and M7 (mg/L)
$CaCl_2$•2 H_2O	293 800	1 000-fold	1.0	293.8
$MgSO_4$•7 H_2O	246 600	2 000-fold	0.5	123.3
KCl	58 000	10 000-fold	0.1	5.8
$NaHCO_3$	64 800	1 000-fold	1.0	64.8
Na_2SiO_3•9 H_2O	50 000	5 000-fold	0.2	10.0
$NaNO_3$	2 740	10 000-fold	0.1	0.274
KH_2PO_4	1 430	10 000-fold	0.1	0.143
$K_2HP\ O_4$	1 840	10 000-fold	0.1	0.184

Table 3. Vitamin stock solutions for medium M4 and M7

	Amount made up to 1 L of water (mg/L)	Concentration (related to medium M4 and M7)	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	10 000-fold		0.075
Cyanocobalamine (B12)	10	10 000-fold	0.1	0.0010
Biotine	7.5	10 000-fold		0.0075

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

ANNEX 4: TOTAL ORGANIC CARBON (TOC) ANALYSIS AND PRODUCTION OF A NOMOGRAPH FOR TOC CONTENT OF ALGAL FEED

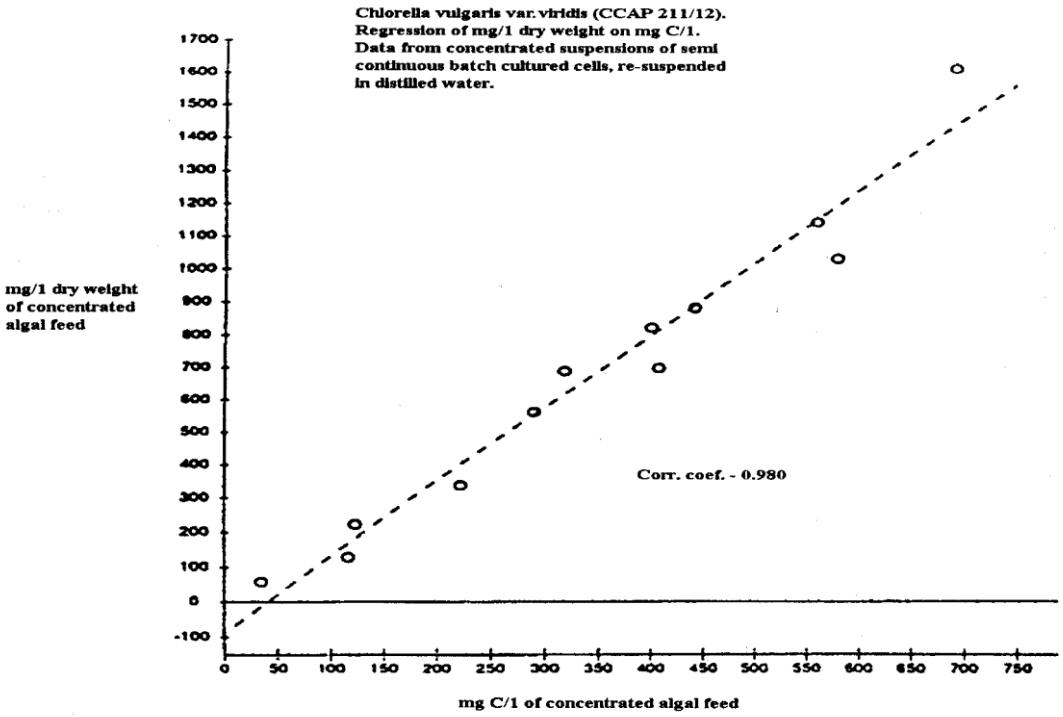
It is recognised that the carbon content of the algal feed will not normally be measured directly but from correlations (i.e. nomographs) with surrogate measures such as algal cell number or light absorbance).

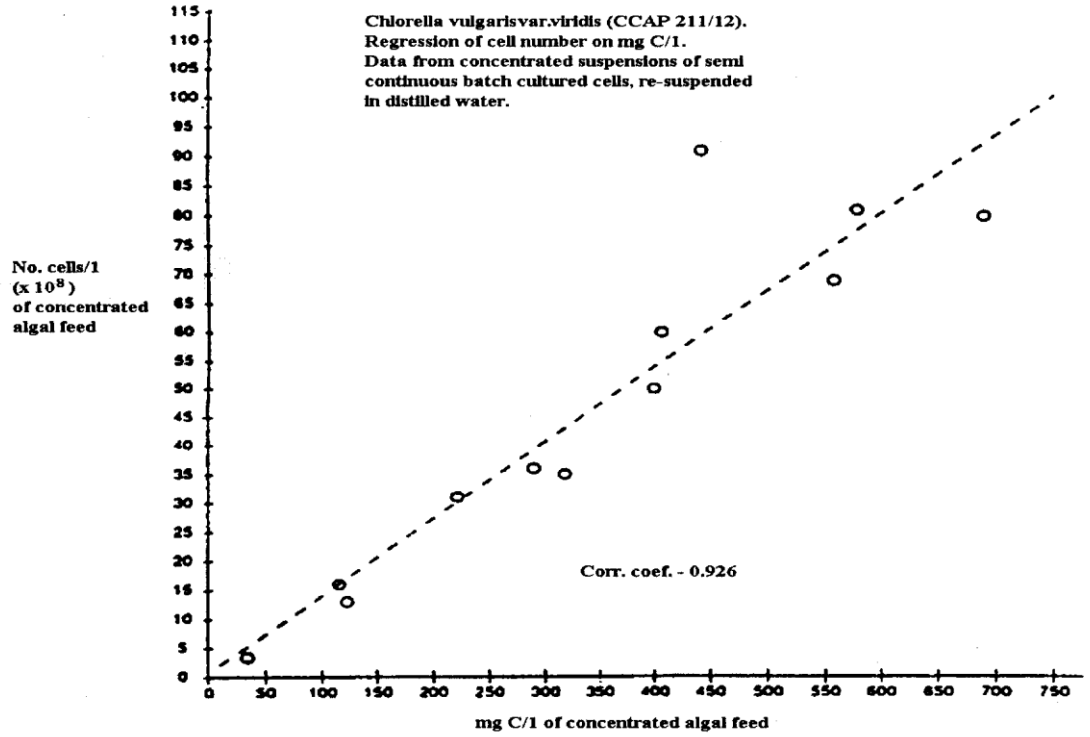
TOC should be measured by high temperature oxidation rather than by UV or persulphate methods. (For advice see: The Instrumental Determination of Total Organic Carbon, Total Oxygen Demand and Related Determinands 1979, HMSO 1980; 49 High Holborn, London WC1V 6HB).

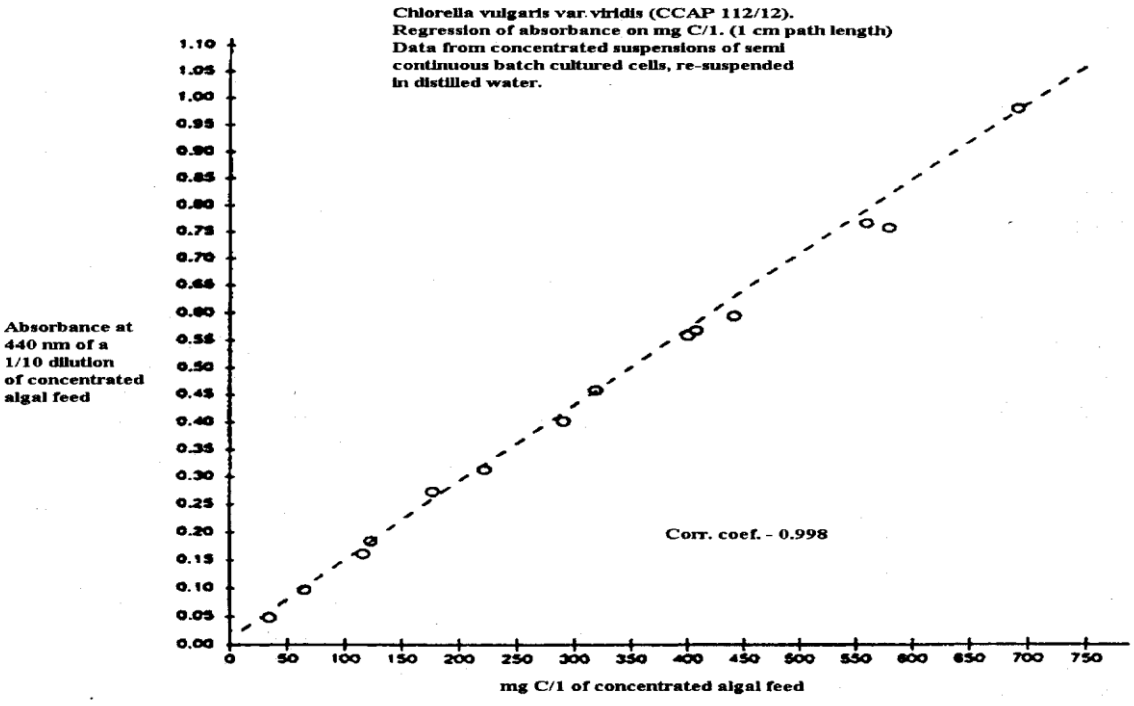
For nomograph production, algae should be separated from the growth medium by centrifugation followed by resuspension in distilled water. Measure the surrogate parameter and TOC concentration in each sample in triplicate. Distilled water blanks should be analysed and the TOC concentration deducted from that of the algal sample TOC concentration.

Nomographs should be linear over the required range of carbon concentrations. Examples are shown below.

N.B. THESE SHOULD NOT BE USED FOR CONVERSIONS; IT IS ESSENTIAL THAT LABORATORIES PREPARE THEIR OWN NOMOGRAPHS.


Chlorella vulgaris var. viridis (CCAP 211/12).
Regression of mg/1 dry weight on mg C/1.
Data from concentrated suspensions of semi continuous batch cultured cells, re-suspended in distilled water.
mg/1 dry weight of concentrated algal feed
Corr. coef. - 0.980
mg C/1 of concentrated algal feed


Chlorella vulgarisvar.viridis (CCAP 211/12).
Regression of cell number on mg C/1.
Data from concentrated suspensions of semi continuous batch cultured cells, re-suspended in distilled water.
No. cells/1 (x 10⁸) of concentrated algal feed
Corr. coef. - 0.926
mg C/1 of concentrated algal feed


Chlorella vulgaris var.viridis (CCAP 112/12).
Regression of absorbance on mg C/1. (1 cm path length)
Data from concentrated suspensions of semi continuous batch cultured cells, re-suspended in distilled water.
Absorbance at 440 nm of a 1/10 dilution of concentrated algal feed
Corr. coef. - 0.998
mg C/1 of concentrated algal feed

ANNEX 5: CALCULATION OF A TIME-WEIGHTED MEAN

Time-weighted mean

Given that the concentration of the test chemical can decline over the period between medium renewals, it is necessary to consider what concentration should be chosen as representative of the range of concentrations experienced by the parent *Daphnia*. The selection should be based on biological considerations as well as statistical ones. For example, if male offspring induction is thought to be affected mostly by the peak concentration experienced, then the maximum concentration should be used. However, if the accumulated or longer term effect of the toxic chemical is considered to be more important, then an average concentration is more relevant. In this case, an appropriate average to use is the time-weighted mean concentration, since this takes account of the variation in instantaneous concentration over time.

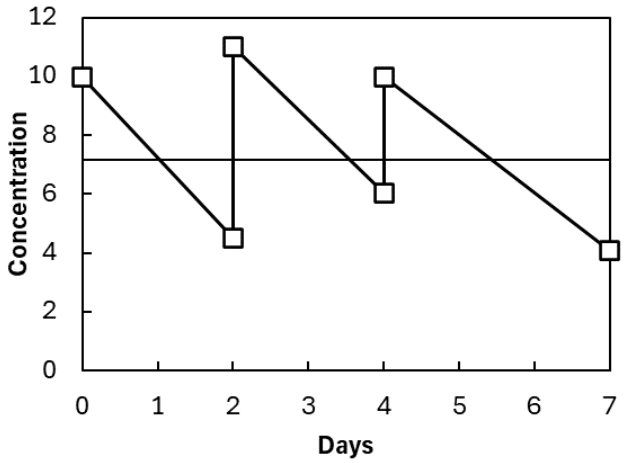


Figure 1: Example of time-weighted mean

Figure 1 shows an example of a (simplified) test lasting seven days with medium renewal at Days 0, 2 and 4.

- The thin zig-zag line represents the concentration at any point in time. The fall in concentration is assumed to follow an exponential decay process.
- The 6 plotted points represent the observed concentrations measured at the start and end of each renewal period.
- The thick solid line indicates the position of the time-weighted mean.

The time-weighted mean is calculated so that the area under the time-weighted mean is equal to the area under the concentration curve. The calculation for the above example is illustrated in Table 1.

Table 1: Calculation of Time-weighted mean

Renewal No.	Days	Conc 0	Conc 1	Ln (Conc 0)	Ln (Conc 1)	Area
1	2	10.000	4.493	2.303	1.503	13.766
2	2	11.000	6.037	2.398	1.798	16.544
3	3	10.000	4.066	2.303	1.403	19.782
Total Days:	7				Total Area:	50.092
					TW mean:	7.156

Days is the number of days in the renewal period
Conc 0 is the measured concentration at the start of each renewal period
Conc 1 is the measured concentration at the end of each renewal period
Ln (Conc 0) is the natural logarithm of Conc 0
Ln (Conc 1) is the natural logarithm of Conc 1

Area is the area under the exponential curve for each renewal period. It is calculated by:

$$Area = \frac{Conc\ 0 - Conc\ 1}{Ln\ (Conc\ 0) - Ln\ (Conc1)} \times Days$$

The time-weighted mean (TW Mean) is the Total Area divided by the Total Days.

It is clear that when observations are taken only at the start and end of each renewal period, it is not possible to confirm that the decay process in, in fact, exponential. A different curve would result in a different calculation for Area. However, an exponential decay process is not implausible and is probably the best curve to use in the absence of other information.

However, a word of caution is required if the chemical analysis fails to find any test chemical at the end of the renewal period. Unless it is possible to estimate how quickly the test chemical disappeared from the solution, it is impossible to obtain a realistic area under the curve, and hence it is impossible to obtain a reasonable time-weighted mean.

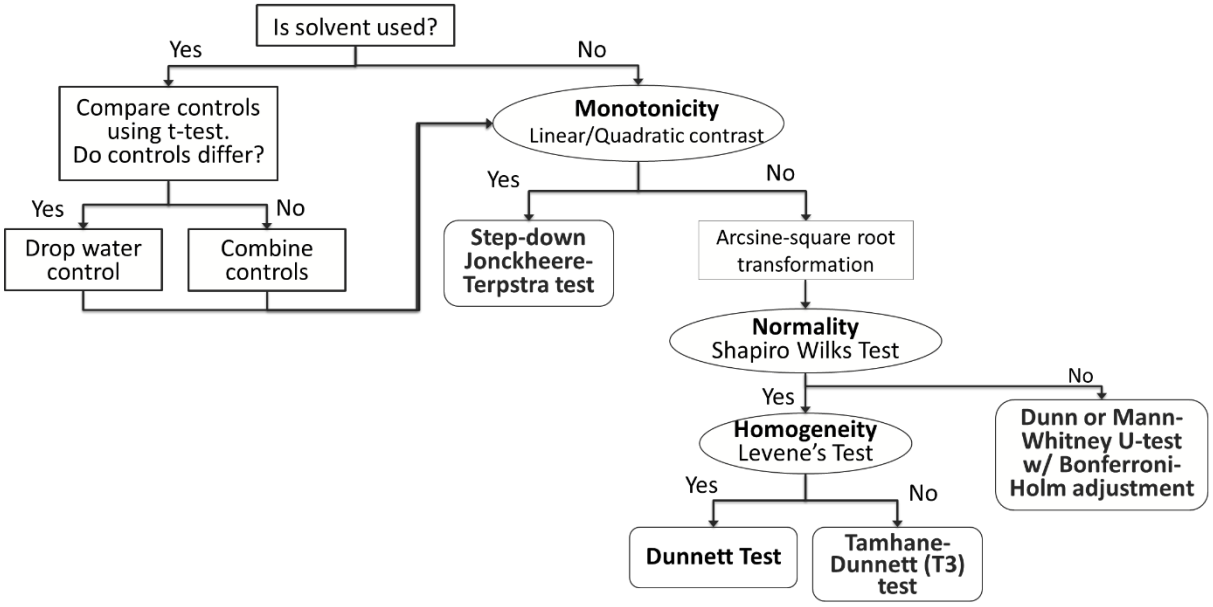
ANNEX 6: STATISTICAL ANALYSIS

The main purpose of this screening test is to identify potential JH activity of the test chemical (1). Thus, TG211 Annex 7 (12) should be used when LOEC or NOEC is demanded. However, if induction of male offspring is also observed in control(s) or the proportion of male offspring in the treatments is not substantial, statistical significance of the proportion of male offspring in the treatments help interpretation of the result.

An example of statistical flow chart for the proportion of male offspring from the JHASA is provided below.
If a solvent is used, a dilution water control and a solvent control should be compared for each response and combined for statistical analysis (e.g. t test) if no significant difference is found between the controls. Otherwise, the solvent control should be used for NOEC determination.

If the data (normally, replicate means) are assumed to be a monotone trend (in an increase or a decrease), it is recommended to analyse the data by a trend-based step-down methods (e.g., step-down Williams test, Jonckheere-Terpstra test). To assess monotonicity, a visual check from a scatter plot can be used, although data should preferably be evaluated by using linear and quadratic contrasts. Unless the quadratic contrast is significant and the linear contrast is not significant, the trend test is done. If the data are non-monotonic, Dunnett's test is used to determine treatment effects if the data show normality and variance homogeneity. Use Tamhane-Dunnett if the data shows normality but heterogeneous variance. If those requirements are not met, then Dunn's test with a Bonferonni-Holm adjustment is used. All indicated tests are done independently of any overall F- or Kruskal-Wallis test. Further details are provided in OECD (2006) (41) and Green et al. (2018) (42).

Alternative methods can be used, such as a generalized linear model (GLM) or generalized liner mixed model (GLMM) with binomial errors for proportion of males, if justified statistically (43). However, because male ratio in the controls is basically zero in all replicates (zero variance), it would be an informal analysis. Step-down Cochran-Armitage test or Fisher's exact test with Bonferroni-Holm adjustment by pooling replicates per treatment is not recommended because sex determination occurs per brood so that the proportion of male should be calculated per replicate.


Is solvent used?
Yes
No
Compare controls using t-test. Do controls differ?
Monotonicity
Linear/Quadratic contrast
Yes
No
Yes
No
Drop water control
Combine controls
Step-down Jonckheere-Terpstra test
Arcsine-square root transformation
Normality
Shapiro Wilks Test
No
Yes
Homogeneity
Levene's Test
Dunn or Mann-Whitney U-test w/ Bonferroni-Holm adjustment
Yes
No
Dunnett Test
Tamhane-Dunnett (T3) test