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COVER NOTE

From:	European Commission
date of receipt:	5 September 2016
To:	General Secretariat of the Council
Subject:	COMMISSION REGULATION (EU) …/… of XXX amending, for the purpose of its adaptation to technical progress, the Annex to Regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)

Delegations will find attached document D045907/02.

Encl.: D045907/02

C.49 Fish Embryo Acute Toxicity (FET) Test

INTRODUCTION

1. This test method (TM) is equivalent to OECD test guideline (TG) 236 (2013). It describes a Fish Embryo Acute Toxicity (FET) test with the zebrafish (*Danio rerio*). This test is designed to determine acute toxicity of chemicals on embryonic stages of fish. The FET-test is based on studies and validation activities performed on zebrafish (1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14). The FET-test has been successfully applied to a wide range of chemicals exhibiting diverse modes of action, solubilities, volatilities, and hydrophobicities (reviewed in 15 and 16).

2. Definitions used in this test method are given in Appendix 1.

PRINCIPLE OF THE TEST

3. Newly fertilised zebrafish eggs are exposed to the test chemical for a period of 96 hrs. Every 24 hrs, up to four apical observations are recorded as indicators of lethality (6): (i) coagulation of fertilised eggs, (ii) lack of somite formation, (iii) lack of detachment of the tail-bud from the yolk sac, and (iv) lack of heartbeat. At the end of the exposure period, acute toxicity is determined based on a positive outcome in any of the four apical observations recorded, and the LC_{50} is calculated.

INITIAL CONSIDERATIONS

4. Useful information about substance-specific properties include the structural formula, molecular weight, purity, stability in water and light, pK_a and K_{ow}, water solubility and vapour pressure as well as results of a test for ready biodegradability (TM C.4 (17) or TM C.29 (18)). Solubility and vapour pressure can be used to calculate Henry's law constant, which will indicate whether losses due to evaporation of the test chemical may occur. A reliable analytical method for the quantification of the substance in the test solutions with known and reported accuracy and limit of detection should be available.

5. If the test method is used for the testing of a mixture, its composition should, as far as possible, be characterised, e.g. by the chemical identity of its constituents, their quantitative occurrence and their substance-specific properties (see paragraph 4). Before use of the test method for regulatory testing of a mixture, it should be considered whether it will provide acceptable results for the intended regulatory purpose.

6. Concerning substances that may be activated via metabolism, there is evidence that zebrafish embryos do have biotransformation capacities (19)(20)(21)(22). However, the metabolic capacity of embryonic fish is not always similar to that of juvenile or adult fish. For instance, the protoxicant allyl alcohol (9) has been missed in the FET. Therefore, if there are any indications that metabolites or other transformation products of relevance may be more toxic than the parent compound, it is also recommended to perform the test with these metabolites/transformation products and to also use these results when concluding on the toxicity of the test chemical, or alternatively perform another test which takes metabolism into further account.

7. For substances with a molecular weight ≥ 3kDa, a very bulky molecular structure, and substances causing delayed hatch which might preclude or reduce the post-hatch exposure, embryos are not expected to be sensitive because of limited bioavailability of the substance, and other toxicity tests might be more appropriate.

VALIDITY OF THE TEST

8. For the test results to be valid, the following criteria apply:

   a) The overall fertilisation rate of all eggs collected should be ≥ 70% in the batch tested.

   b) The water temperature should be maintained at 26 ± 1 °C in test chambers at any time during the test.

   c) Overall survival of embryos in the negative (dilution-water) control, and, where relevant, in the solvent control should be ≥ 90% until the end of the 96 hrs exposure.

   d) Exposure to the positive control (e.g. 4.0 mg/l 3,4-dichloroaniline for zebrafish) should result in a minimum mortality of 30% at the end of the 96 hrs exposure.

   e) Hatching rate in the negative control (and solvent control if appropriate) should be ≥80% at the end of 96 hrs exposure.

   f) At the end of the 96 hrs exposure, the dissolved oxygen concentration in the negative control and highest test concentration should be ≥80% of saturation.

DESCRIPTION OF THE METHOD

9. An overview of recommended maintenance and test conditions is available in Appendix 2.

Apparatus

10. The following equipment is needed:

a) Fish tanks made of chemically inert material (e.g. glass) and of a suitable capacity in relation to the recommended loading (see "Maintenance of brood fish", paragraph 14);

b) Inverted microscope and/or binocular with a capacity of at least 80-fold magnification. If the room used for recording observations cannot be adjusted to 26 ± 1 °C, a temperature-controlled cross movement stage or other methods to maintain temperature are necessary;

c) Test chambers; e.g., standard 24-well plates with a depth of approx. 20 mm. (see "Test chambers", paragraph 11);

d) e.g., self-adhesive foil to cover the 24-well plates;

e) Incubator or air-conditioned room with controlled temperature, allowing to maintain 26 ±1 °C in wells (or test chambers);

f) pH-meter;

g) Oxygen meter;

h) Equipment for determination of hardness of water and conductivity;

i) Spawn trap: instrument trays of glass, stainless steel or other inert materials; wire mesh (grid size 2 ± 0.5 mm) of stainless steel or other inert material to protect the eggs once laid; spawning substrate (e.g. plant imitates of inert material) (TM C.48, Appendix 4a (23));

j) Pipettes with widened openings to collect eggs;

k) Glass vessels to prepare different test concentrations and dilution water (beakers, graduated flasks, graduated cylinders and graduated pipettes) or to collect zebrafish eggs (e.g. beakers, crystallisation dishes);

l) If alternative exposure systems, such as flow-through (24) or passive dosing (25) are used for the conduct of the test, appropriate facilities and equipment are needed.

Test chambers

11. Glass or polystyrene test chambers should be used (e.g. 24-well plates with a 2.5-5 ml filling capacity per well). In case adsorption to polystyrene is suspected (e.g., for

non-polar, planar substances with high K_{OW}), inert materials (glass) should be used to reduce losses due to adsorption (26). Test chambers should be randomly positioned in the incubator.

Water and test conditions

12. Dilution of the maintenance water is recommended to achieve hardness levels typical of a wide variety of surface waters. Dilution water should be prepared from reconstituted water (27). The resulting degree of hardness should be equivalent to 100-300 mg/l $CaCO_3$ in order to prevent excessive precipitation of calcium carbonate. Other well-characterised surface or well water may be used. The reconstituted water may be adapted to maintenance water of low hardness by dilution with deionised water up to a ratio of 1:5 to a minimum hardness of 30-35 mg/l $CaCO_3$. The water is aerated to oxygen saturation prior to addition of the test chemical. Temperature should be kept at 26 ± 1°C, in the wells, throughout the test. The pH should be in a range between pH 6.5 and 8.5, and not vary within this range by more than 1.5 units during the course of the test. If the pH is not expected to remain in this range, then pH adjustment should be done prior to initiating the test. The pH adjustment should be made in such a way that the stock solution concentration is not changed to any significant extent and that no chemical reaction or precipitation of the test chemical is caused. Use of hydrogen chloride (HCl) and sodium hydroxide (NaOH) to correct pH in the solutions containing the test chemical is recommended.

Test solutions

13. Test solutions of the selected concentrations can be prepared, e.g. by dilution of a stock solution. The stock solutions should preferably be prepared by simply mixing or agitating the test chemical in the dilution water by mechanical means (e.g. stirring and/or ultra-sonification). If the test chemical is difficult to dissolve in water, procedures described in the OECD Guidance Document No. 23 for handling difficult substances and mixtures should be followed (28). The use of solvents should be avoided, but may be required in some cases in order to produce a suitably concentrated stock solution. Where a solvent is used to assist in stock solution preparation, its final concentration should not exceed 100 µl/l and should be the same in all test vessels. When a solvent is used, an additional solvent control is required.

Maintenance of brood fish

14. A breeding stock of unexposed, wild-type zebrafish with well-documented fertilisation rate of eggs is used for egg production. Fish should be free of macroscopically discernible symptoms of infection and disease and should not have

undergone any pharmaceutical (acute or prophylactic) treatment for 2 months before spawning. Breeding fish are maintained in aquaria with a recommended loading capacity of 1 l water per fish and a fixed 12 – 16 hour photoperiod (29)(30)(31)(32)(33). Optimal filtering rates should be adjusted; excess filtering rates causing heavy perturbation of the water should be avoided. For feeding conditions, see Appendix 2. Surplus feeding should be avoided, and water quality and cleanness of the aquaria should be monitored regularly and be reset to the initial state, if necessary.

Proficiency Testing

15. As a reference chemical, 3,4-dichloroaniline (used in the validation studies (1)(2)), should be tested in a full concentration-response range to check the sensitivity of the fish strain used, preferably twice a year. For any laboratory initially establishing this assay, the reference chemical should be used. A laboratory can use this chemical to demonstrate their technical competence in performing the assay prior to submitting data for regulatory purposes.

Egg production

16. Zebrafish eggs may be produced via spawning groups (in individual spawning tanks) or via mass spawning (in the maintenance tanks). In the case of spawning groups, males and females (e.g., at a ratio of 2:1) in a breeding group are placed in spawning tanks a few hours before the onset of darkness on the day prior to the test. Since spawning groups of zebrafish may occasionally fail to spawn, the parallel use of at least three spawning tanks is recommended. To avoid genetic bias, eggs are collected from a minimum of three breeding groups, mixed and randomly selected.

17. For the collection of eggs, spawn traps are placed into the spawning tanks or maintenance tanks before the onset of darkness on the day prior to the test or before the onset of light on the day of the test. To prevent predation of eggs by adult zebrafish, the spawn traps are covered with inert wire mesh of appropriate mesh size (approx. 2 ±0.5 mm). If considered necessary, artificial plants made of inert material (e.g., plastic or glass) can be fixed to the mesh as spawning stimulus (3)(4)(5)(23)(35). Weathered plastic materials which do not leach (e.g., phthalates) should be used. Mating, spawning and fertilisation take place within 30 min after the onset of light and the spawn traps with the collected eggs can be carefully removed. Rinsing eggs with reconstituted water after collection from spawning traps is recommended.

Egg differentiation

18. At 26°C, fertilised eggs undergo the first cleavage after about 15 min and the consecutive synchronous cleavages form 4, 8, 16 and 32 cell blastomers (see Appendix 3)(35). At these stages, fertilised eggs can be clearly identified by the development of a blastula.

PROCEDURE

Conditions of exposure

19. Twenty embryos per concentration (one embryo per well) are exposed to the test chemical. Exposure should be such that ±20% of the nominal chemical concentration are maintained throughout the test. If this is not possible in a static system, a manageable semi-static renewal interval should be applied (e.g. renewal every 24 hrs). In these cases exposure concentrations need to be verified as a minimum in the highest and lowest test concentrations at the beginning and the end of each exposure interval (see paragraph 36). If an exposure concentration of ±20% of the nominal concentrations cannot be maintained, all concentrations need to be measured at the beginning and the end of each exposure interval (see paragraph 36). Upon renewal, care should be taken that embryos remain covered by a small amount of old test solutions to avoid drying. The test design can be adapted to meet the testing requirements of specific substances (*e.g,.* flow-through (24) or passive dosing systems (25) for easily degradable or highly adsorptive substances (29), or others for volatile substances (36)(37)). In any case, care should be taken to minimise any stress to the embryos. Test chambers should be conditioned at least for 24 hrs with the test solutions prior to test initiation. Test conditions are summarised in Appendix 2.

Test concentrations

20. Normally, five concentrations of the test chemical spaced by a constant factor not exceeding 2.2 are required to meet statistical requirements. Justification should be provided, if fewer than five concentrations are used. The highest concentration tested should preferably result in 100% lethality, and the lowest concentration tested should preferably give no observable effect, as defined in paragraph 28. A range-finding test before the definitive test allows selection of the appropriate concentration range. The range-finding is typically performed using ten embryos per concentration. The following instructions refer to performing the test in 24-well plates. If different test

chambers (e.g. small Petri dishes) are used or more concentrations are tested, instructions have to be adjusted accordingly.

21. Details and visual instructions for allocation of concentrations across 24-well plates are available in paragraph 27 and Appendix 4, Figure 1.

Controls

22. Dilution water controls are required both as negative control and as internal plate controls. If more than 1 dead embryo is observed in the internal plate control, the plate is rejected, thus reducing the number of concentrations used to derive the LC_{50}. If an entire plate is rejected the ability to evaluate and discern observed effects may become more difficult, especially if the rejected plate is the solvent control plate or a plate in which treated embryos are also affected. In the first case the test must be repeated. In the second one the loss of an entire treatment group(s) due to internal control mortality may limit the ability to evaluate effects and determine LC_{50} values.

23. A positive control at a fixed concentration of 4 mg/l 3,4-dichloroaniline is performed with each egg batch used for testing.

24. In case a solvent is used, an additional group of 20 embryos is exposed to the solvent on a separate 24-well plate, thus serving as a solvent control. To consider the test acceptable, the solvent should be demonstrated to have no significant effects on time to hatch, survival, nor produce any other adverse effects on the embryos (cf. paragraph 8c).

Start of exposure and duration of test

25. The test is initiated as soon as possible after fertilisation of the eggs and terminated after 96 hrs of exposure. The embryos should be immersed in the test solutions before cleavage of the blastodisc commences, or, at latest, by the 16 cell-stage. To start exposure with minimum delay, at least twice the number of eggs needed per treatment group are randomly selected and transferred into the respective concentrations and controls (e.g. in 100 ml crystallisation dishes; eggs should be fully covered) not later than 90 minutes post fertilisation.

26. Viable fertilised eggs should be separated from unfertilised eggs and be transferred to 24-well plates pre-conditioned for 24 hrs and refilled with 2 ml/well freshly prepared test solutions within 180 minutes post fertilisation. By means of stereomicroscopy (preferably ≥30-fold magnification), fertilised eggs undergoing cleavage and showing no obvious irregularities during cleavage (e.g. asymmetry, vesicle formation) or injuries of the chorion are selected. For egg collection and separation, see Appendix 3, Fig. 1 and 3 and Appendix 4, Fig. 2.

Distribution of eggs over the 24-well plates

27. Eggs are distributed to well plates in the following numbers (see also Appendix 4, Fig. 1)

    - 20 eggs on one plate for each test concentration;
    - 20 eggs as solvent control on one plate (if necessary);
    - 20 eggs as positive control on one plate;
    - 4 eggs in dilution water as internal plate control on each of the above plates;
    - 24 eggs in dilution water as negative control on one plate.

Observations

28. Apical observations performed on each tested embryo include: coagulation of embryos, lack of somite formation, non-detachment of the tail, and lack of heartbeat (Table 1). These observations are used for the determination of lethality: Any positive outcome in one of these observations means that the zebrafish embryo is dead. Additionally, hatching is recorded in treatment and control groups on a daily basis starting from 48 hrs. Observations are recorded every 24 hrs, until the end of the test.

Table 1. Apical observations of acute toxicity in zebrafish embryos 24-96 hrs post fertilisation.

	Exposure times			
	24 hrs	48 hrs	72 hrs	96 hrs
Coagulated embryos	+	+	+	+
Lack of somite formation	+	+	+	+
Non-detachment of the tail	+	+	+	+
Lack of heartbeat		+	+	+

29. *Coagulation of the embryo:* Coagulated embryos are milky white and appear dark under the microscope (see Appendix 5, Fig. 1). The number of coagulated embryos is determined after 24, 48, 72 and 96 hrs.

30. *Lack of somite formation:* At 26 ±1°C, about 20 somites have formed after 24 hrs (see Appendix 5, Figure 2) in a normally developing zebrafish embryo. A normally developed embryo shows spontaneous movements (side-to-side contractions). Spontaneous movements indicate the formation of somites. The absence of somites is recorded after 24, 48, 72 and 96 hrs. Non-formation of somites after 24 hrs might be due to a general retardation of development. At latest after 48 hrs, the formation of somites should be developed. If not, the embryos are considered dead.

31. *Non-detachment of the tail:* In a normally developing zebrafish embryo, detachment of the tail (seeAppendix 5, Figure 3) from the yolk is observed following posterior elongation of the embryonic body. Absence of tail detachment is recorded after 24, 48, 72 and 96 hrs.

32. *Lack of heartbeat:* In a normally developing zebrafish embryo at 26 ±1°C, the heartbeat is visible after 48 hrs (see Appendix 5, Figure 4). Particular care should be taken when recording this endpoint, since irregular (erratic) heartbeat should *not* be recorded as lethal. Moreover, visible heartbeat without circulation in aorta abdominalis is considered non-lethal. To record this endpoint, embryos showing no heartbeat should be observed under a minimum magnification of 80x for at least one minute. Absence of heartbeat is recorded after 48, 72 and 96 hrs.

33. Hatching rates of all treatment and control groups should be recorded from 48 hrs onwards and reported. Although hatching is not an endpoint used for the calculation of the LC_{50}, hatching ensures exposure of the embryo without a potential barrier function of the chorion, and as such may help data interpretation.

34. Detailed descriptions of the normal (35) and examples of abnormal development of zebrafish embryos are illustrated in Appendixes 3 and 5.

Analytical measurements

35. At the beginning and at the end of the test, pH, total hardness and conductivity in the control(s) and in the highest test chemical concentration are measured. In semi-static renewal systems the pH should be measured prior to and after water renewal. The dissolved oxygen concentration is measured at the end of the test in the negative controls and highest test concentration with viable embryos, where it should be in compliance with the test validity criteria (see paragraph 8f). If there is concern that the temperature varies across the 24-well plates, temperature is measured in three randomly selected vessels. Temperature should be recorded preferably continuously during the test or, as a minimum, daily.

36. In a static system, the concentration of the test chemical should be measured, as a minimum, in the highest and lowest test concentrations, but preferably in all treatments, at the beginning and end of the test. In semi-static (renewal) tests where the concentration of the test chemical is expected to remain within ± 20% of the nominal values, it is recommended that, as a minimum, the highest and lowest test concentrations be analysed when freshly prepared and immediately prior to renewal. For tests where the concentration of the test chemical is not expected to remain within ± 20% of nominal, all test concentrations must be analysed when freshly prepared and immediately prior to renewal. In case of insufficient volume for analysis, merging of test solutions, or use of surrogate chambers being of the same

material and having the same volume to surface area ratios as 24-well plates, may be useful. It is strongly recommended that results be based on measured concentrations. When the concentrations do not remain within 80-120% of the nominal concentration, the effect concentrations should be expressed relative to the geometric mean of the measured concentrations; see Chapter 5 in the OECD Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures for more details (28).

LIMIT TEST

37. Using the procedures described in this test method, a limit test may be performed at 100 mg/l of test chemical or at its limit of solubility in the test medium (whichever is the lower) in order to demonstrate that the LC_{50} is greater than this concentration. The limit test should be performed using 20 embryos in the treatment, the positive control and –if necessary- in the solvent control and 24 embryos in the negative control. If the percentage of lethality at the concentration tested exceeds the mortality in the negative control (or solvent control) by 10%, a full study should be conducted. Any observed effects should be recorded. If mortality exceeds 10% in the negative control (or solvent control), the test becomes invalid and should be repeated.

DATA AND REPORTING

Treatment of results

38. In this test, the individual wells are considered independent replicates for statistical analysis. The percentages of embryos for which at least one of the apical observations is positive at 48 and/or 96 hrs are plotted against test concentrations. For calculation of the slopes of the curve, LC_{50} values and the confidence limits (95%), appropriate statistical methods should be applied (38) and the OECD Guidance Document on Current Approaches in the Statistical Analysis of Ecotoxicity Data should be consulted (39).

Test report

39. The test report should include the following information:

Test chemical:

Mono-constituent substance

- physical appearance, water solubility, and additional relevant physicochemical properties;

- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate).

Multi-constituent substance, UVCBs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Test organisms:

- scientific name, strain, source and method of collection of the fertilised eggs and subsequent handling.

Test conditions:

- test procedure used (e.g., semi-static renewal);
- photoperiod;
- test design (e.g., number of test chambers, types of controls);
- water quality characteristics in fish maintenance (e.g. pH, hardness, temperature, conductivity, dissolved oxygen);
- dissolved oxygen concentration, pH, total hardness, temperature and conductivity of the test solutions at the start and after 96 hrs;
- method of preparation of stock solutions and test solutions as well as frequency of renewal;
- justification for use of solvent and justification for choice of solvent, if other than water;
- the nominal test concentrations and the result 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 quantification (LoQ) should also be reported;
- evidence that controls met the overall survival validity criteria;
- fertilisation rate of the eggs;
- hatching rate in treatment and control groups.

Results:

- maximum concentration causing no mortality within the duration of the test;
- minimum concentration causing 100% mortality within the duration of the test;
- cumulative mortality for each concentration at the recommended observation times;
- the LC_{50} values at 96 hrs (and optionally at 48 hrs) for mortality with 95% confidence limits, if possible;

- graph of the concentration-mortality curve at the end of the test;
- mortality in the controls (negative controls, internal plate controls, as well as positive control and any solvent control used);
- data on the outcome of each of the four apical observations;
- incidence and description of morphological and physiological abnormalities, if any (see examples provided in Appendix 5, Figure 2);
- incidents in the course of the test which might have influenced the results;
- statistical analysis and treatment of data (probit analysis, logistic regression model and geometric mean for LC_{50});
- slope and confidence limits of the regression of the (transformed) concentration-response curve.

Any deviation from the test method and relevant explanations.

Discussion and interpretation of results.

LITERATURE

(1) OECD (2011) Validation Report (Phase 1) for the Zebrafish Embryo Toxicity Test: Part I and Part II. Series on Testing and Assessment No. 157, OECD, Paris.

(2) OECD (2012) Validation Report (Phase 2) for the Zebrafish Embryo Toxicity Test: Part I and Part II (Annexes). Series on Testing and Assessment No. 179, OECD, Paris.

(3) Braunbeck, T., Böttcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., Rudolf, M. and Seitz, N. (2005) Towards an alternative for the acute fish LC_{50} test in chemical assessment: The fish embryo toxicity test goes multi-species-an update. ALTEX 22: 87-102.

(4) ISO (2007) International Standard Water quality – Determination of the acute toxicity of waste water to zebrafish eggs (*Danio rerio*). ISO 15088:2007(E) International Organization for Standardization.

(5) Nagel, R. (2002) DarT: The embryo test with the zebrafish (*Danio rerio*) - a general model in ecotoxicology and toxicology. ALTEX 19: 38-48.

(6) Schulte, C. and Nagel, R. (1994) Testing acute toxicity in embryo of zebrafish, *Brachydanio rerio* as alternative to the acute fish test - preliminary results. ATLA 22, 12-19.

(7) Bachmann, J. (2002) Development and validation of a teratogenicity screening test with embryos of the zebrafish (*Danio rerio*). PhD-thesis, Technical University of Dresden, Germany.

(8) Lange, M., Gebauer, W., Markl, J. and Nagel, R. (1995) Comparison of testing acute toxicity on embryo of zebrafish (*Brachydanio rerio*), and RTG-2 cytotoxicity as possible alternatives to the acute fish test. Chemosphere 30/11: 2087-2102.

(9) Knöbel, M., Busser, F.J.M., Rico-Rico, A., Kramer, N.I., Hermens, J.L.M., Hafner, C., Tanneberger, K., Schirmer, K., Scholz, S. (2012). Predicting adult fish acute lethality with the zebrafish embryo: relevance of test duration, endpoints, compound properties, and exposure concentration analysis. Environ. Sci. Technol. 46, 9690-9700.

(10) Kammann, U., Vobach, M. and Wosniok, W. (2006) Toxic effects of brominated indoles and phenols on zebrafish embryos. Arch. Environ. Contam. Toxicol., 51: 97-102.

(11) Groth, G., Kronauer, K. and Freundt, K.J. (1994) Effects of *N,N*-diemethylformamide and its degradation products in zebrafish embryos. Toxicol. *In Vitro* 8: 401-406.

(12) Groth, G., Schreeb, K., Herdt, V. and Freundt, K.J. (1993) Toxicity studies in fertilized zebrafish fish eggs treated with *N*-methylamine, *N,N*-dimethylamine, 2-aminoethanol, isopropylamine, aniline, *N*-methylaniline, *N,N*-dimethylaniline, quinone, chloroacetaldehyde, or cyclohexanol. Bull. Environ. Contam. Toxicol. 50: 878-882.

(13) Nguyen, L.T. and Janssen, C.R. (2001) Comparative sensitivity of embryo-larval toxicity assays with African catfish (*Clarias gariepinus*) and zebra fish (*Danio rerio*). Environ. Toxicol. 16: 566-571.

(14) Cheng, S.H., Wai, A.W.K., So, C.H. and Wu, R.S.S. (2000) Cellular and molecular basis of cadmium-induced deformities in zebrafish embryos. Environ. Toxicol. Chem. 19: 3024-3031.

(15) Belanger, S. E., Rawlings J. M. and Carr G. J. (2013). Use of Fish Embryo Toxicity Tests for the Prediction of Acute Fish Toxicity to Chemicals. Environmental Toxicology and Chemistry 32: 1768-1783. .

(16) Lammer, E., Carr, G. J., Wendler, K., Rawlings, J. M., Belanger, S. E., Braunbeck, T. (2009) Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? Comp. Biochem. Physiol. C Toxicol. Pharmacol.: 149 (2), 196−209

(17) Chapter C.4 of this Annex: Ready Biodegradability.

(18) Chapter C.29 of this Annex: Ready Biodegradability, CO_2 in sealed vessels.

(19) Weigt, S., Huebler, N., Strecker, R., Braunbeck, T., Broschard, T.H. (2011) Zebrafish (*Danio rerio*) embryos as a model for testing proteratogens. Toxicology 281: 25-36.

(20) Weigt, S., Huebler, N., Strecker, R., Braunbeck, T., Broschard, T.H. (2012) Developmental effects of coumarin and the anticoagulant coumarin derivative warfarin on zebrafish (*Danio rerio*) embryos. Reprod. Toxicol. 33: 133-141.

(21) Incardona, J.P, Linbo, T.L., Scholz, N.L. (2011) Cardiac toxicity of 5-ring polycyclic aromatic hydrocarbons is differentially dependent on the

aryl hydrocarbon receptor 2 isoform during zebrafish development. Toxicol. Appl. Pharmacol. 257: 242-249.

(22) Kubota, A., Stegeman, J.J., Woodin, B.R., Iwanaga, T., Harano, R., Peterson, R.E., Hiraga, T., Teraoka, H. (2011) Role of zebrafish cytochrome P450 CYP1C genes in the reduced mesencephalic vein blood flow caused by activation of AHR2. Toxicol. Appl. Pharmacol. 253: 244-252.

(23) Chapter C.48 of this Annex: Fish Short Term Reproduction Assay.. See Appendix 4a.

(24) Lammer, E., Kamp, H.G., Hisgen, V., Koch, M., Reinhard, D., Salinas, E.R., Wendlar, K., Zok, S., Braunbeck, T. (2009) Development of a flow-through system for the fish embryo toxicity test (FET) with zebrafish (*Danio rerio*). Toxicol. *in vitro* 23: 1436-1442.

(25) Brown, R.S., Akhtar, P., Åkerman, J., Hampel, L., Kozin, I.S., Villerius, L.A., Klamer, H.J.C., (2001) Partition controlled delivery of hydrophobic substances in toxicity tests using poly(dimethylsiloxane) (PDMS) films. Environ. Sci. Technol. 35, 4097–4102.

(26) Schreiber, R., Altenburger, R., Paschke, A., Küster, E. (2008) How to deal with lipophilic and volatile organic substances in microtiter plate assays. Environ. Toxicol. Chem. 27, 1676-1682.

(27) ISO (1996) International Standards. Water quality - Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)]. ISO 7346-3: Flow-through method. Available: [http://www.iso.org].

(28) OECD (2000) Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. Series on Testing and Assessment No. 23, OECD, Paris.

(29) Laale, H.W. (1977) The biology and use of zebrafish, *Brachydanio rerio*, in fisheries research. A literature review. J. Fish Biol. 10: 121-173.

(30) Westerfield, M. (2007) The zebrafish book: A guide for the laboratory use of zebrafish (*Brachydanio rerio*). 5th edition. Eugene, University of Oregon Press, Institute of Neuroscience, USA.

(31) Canadian Council on Animal Care (2005) Guidelines on: the Care and Use of Fish in Research, Teaching and Testing, ISBN: 0-919087-43-4 http://www.ccac.ca/Documents/Standards/Guidelines/Fish.pdf

(32) European Commission (2007) Commission recommendation 2007/526/EC of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes (notified under document number C(2007) 2525) [http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:197:0001:0089:EN:PDF]

(33) European Union (2010) Directive 2010/63/EU of the European Parliament and Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal of the European Union, L 276:33-79; 20.10.2010 http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:EN:PDF

(34) Nagel, R. (1986) Untersuchungen zur Eiproduktion beim Zebrabärbling (*Brachydanio rerio*, Ham.-Buch.). J. Appl. Ichthyol. 2: 173-181.

(35) Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B. and Schilling, T.F. (1995) Stages of embryonic development of the zebrafish. Dev. Dyn. 203: 253-310.

(36) Chapter C.2 of this Annex: Daphnia sp., Acute Immobilistaion Test.

(37) Weil, M., Scholz, S., Zimmer, M., Sacher, F., Duis, K. (2009) Gene expression analysis in zebrafish embryos: a potential approach to predict effect conentrations in the fish early life stage test. Environ. Toxicol. Chem. 28: 1970-1978

(38) ISO (2006) International Standard. Water quality - Guidance on statistical interpretation of ecotoxicity data ISO TS 20281. Available: [http://www.iso.org].

(39) OECD (2006) Guidance Document on Current Approaches in the Statistical Analysis of Ecotoxicity Data: a Guidance to Application. Series on Testing and Assessment No. 54. OECD, Paris.

(40) Braunbeck, T., Lammer, E., 2006. Detailed review paper “Fish embryo toxicity assays”. UBA report under contract no. 20385422 German Federal Environment Agency, Berlin. 298 pp.

Appendix 1

DEFINITIONS

Apical endpoint: Causing effect at population level.

Blastula: A cellular formation around the animal pole that covers a certain part of the yolk.

Chemical: A substance or a mixture

Epiboly: is a massive proliferation of predominantly epidermal cells in the gastrulation phase of the embryo and their movement from the dorsal to the ventral side, by which entodermal cell layers are internalised in an invagination-like process and the yolk is incorporated into the embryo.

Flow-through test: A test with continued flow of test solutions through the test system during the duration of exposure.

Internal Plate Control: Internal control consisting of 4 wells filled with dilution water per 24-well plate to identify potential contamination of the plates by the manufacturer or by the researcher during the procedure, and any plate effect possibly influencing the outcome of the test (e.g. temperature gradient).

IUPAC: International Union of Pure and Applied Chemistry

Maintenance water: Water in which the husbandry of the adult fish is performed.

Median Lethal Concentration (LC_{50}): The concentration of a test chemical that is estimated to be lethal to 50% of the test organisms within the test duration.

Semi-static renewal test: A test with regular renewal of the test solutions after defined periods (e.g., every 24 hrs).

SMILES: Simplified Molecular Input Line Entry Specification

Somite: In the developing vertebrate embryo, somites are masses of mesoderm distributed laterally to the neural tube, which will eventually develop dermis (dermatome), skeletal muscle (myotome), and vertebrae (sclerotome).

Static test: A test in which test solutions remain unchanged throughout the duration of the test.

Test chemical: Any substance or mixture tested using this test method

UVCB: Substances of unknown or variable composition, complex reaction products or biological materials

Appendix *2*

MAINTENANCE, BREEDING AND TYPICAL CONDITIONS FOR ZEBRAFISH EMBRYO ACUTE TOXICITY TESTS

Zebrafish (*Danio rerio*)		
Origin of species		India, Burma, Malakka, Sumatra
Sexual dimorphism		Females: protruding belly, when carrying eggs Males: more slender, orange tint between blue longitudinal stripes (particularly evident at the anal fin)
Feeding regime		Dry flake food (max. 3% fish weight per day) 3 - 5 times daily; additionally brine shrimp (*Artemia* spec.) *nauplii* and / or small daphnids of appropriate size obtained from an uncontaminated source. Feeding live food provides a source of environmental enrichment and therefore live food should be given wherever possible. To guarantee for optimal water quality, excess food and faeces should be removed approx. one hour after feeding.
Approximate weight of adult fish		Females:0.65 ±0.13 g Males: 0.5 ±0.1 g
Maintenance of parental fish	Illumination	Fluorescent bulbs (wide spectrum); 10-20 µE/m^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels); 12-16 hrs photoperiod
	Water temperature	26 ±1 °C
	Water quality	O_2 ≥80% saturation, hardness: e.g. ~30-300 mg/l $CaCO_3$, NO_3^- : ≤48mg/l, NH_4^+ and NO_2^-: <0.001 mg/l, residual chlorine <10 µg/l, total organic chlorine <25 ng/l, pH = 6.5 - 8.5
	Further water quality criteria	Particulate matter <20 mg/l, total organic carbon <2 mg/l, total organophosphorus pesticides <50 ng/l, total organochlorine pesticides plus polychlorinated biphenyls <50 ng/l
	Tank size for maintenance	e.g. 180 l, 1 fish/l
	Water purification	Permanent (charcoal filtered); other possibilities include combinations with semi-static renewal maintenance or flow-through system with continuous water renewal
Recommended male to female ratio for breeding		2:1 (or mass spawning)
Spawning tanks		e.g. 4 l tanks equipped with steel grid bottom and plant dummy as spawning stimulant; external heating mats, or mass spawning within the maintenance tanks

Egg structure and appearance	Stable chorion (*i.e.* highly transparent, non-sticky, diameter ~ 0.8–1.5 mm)
Spawning rate	A single mature female spawns at least 50-80 eggs per day. Depending on the strain, spawning rates may be considerably higher. The fertilisation rate should be ≥70%. For first time spawning fish, fertilisation rates of the eggs may be lower in the first few spawns.
Test type	Static, semi-static renewal, flow-through, 26 ±1°C, 24 hrs conditioned test chambers (e.g. 24-well plates 2.5-5 ml per cavity)

Appendix *3*

NORMAL ZEBRAFISH DEVELOPMENT AT 26°C

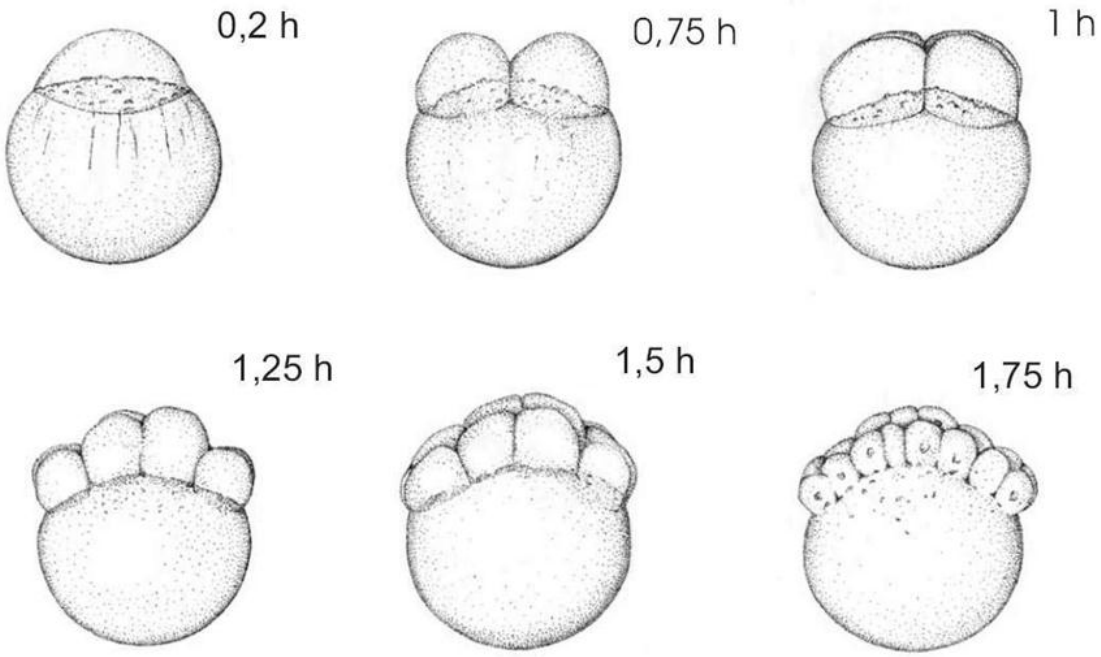


Fig. 1: Selected stages of early zebrafish (Danio rerio) development: 0.2 – 1.75 hrs post-fertilisation (from Kimmel *et al*., 1995 (35)). The time sequence of normal development may be taken to diagnose both fertilisation and viability of eggs (see paragraph 26: Selection of fertilised eggs).

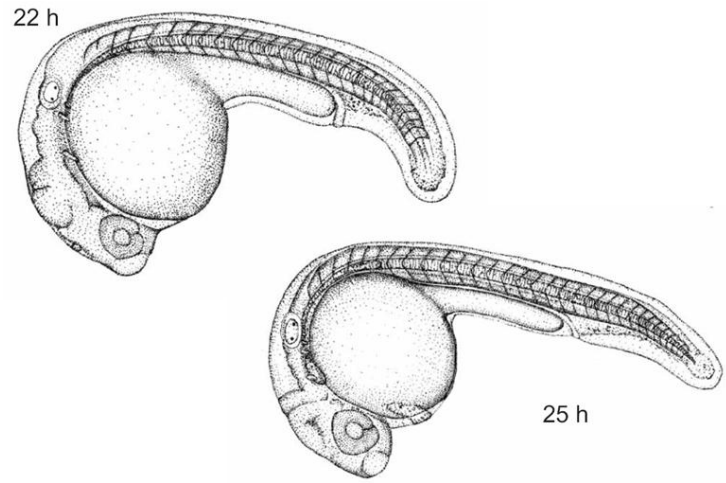
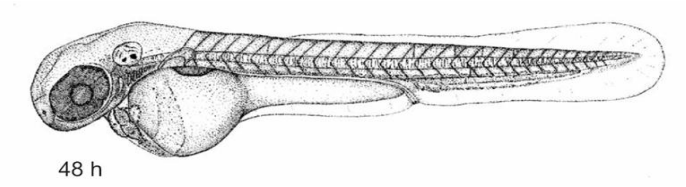


Fig. 2: Selected stages of late zebrafish (Danio rerio) development (de-chorionated embryo to optimise visibility): 22 - 48 hrs after fertilisation (from Kimmel *et al*., 1995 (35)).



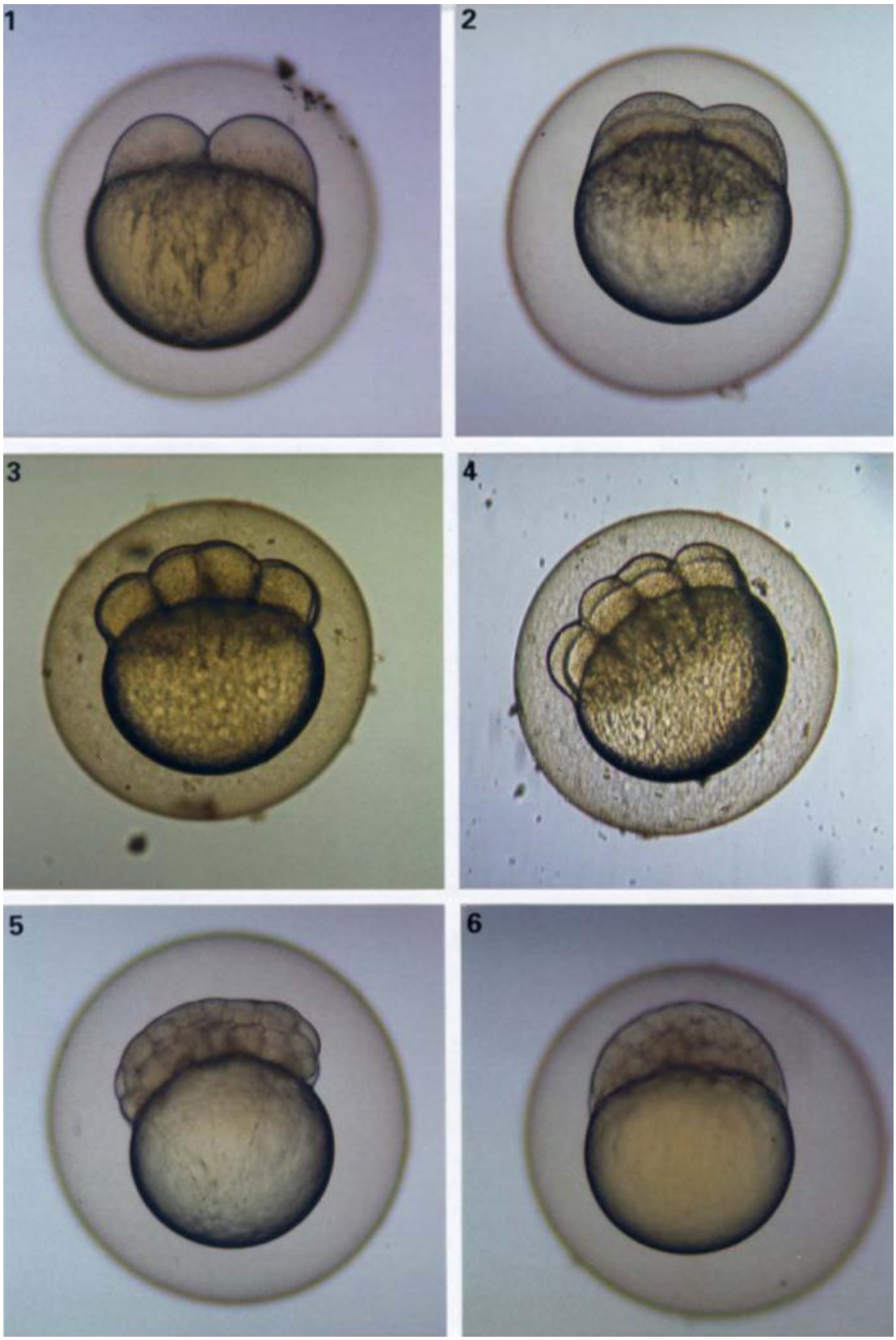
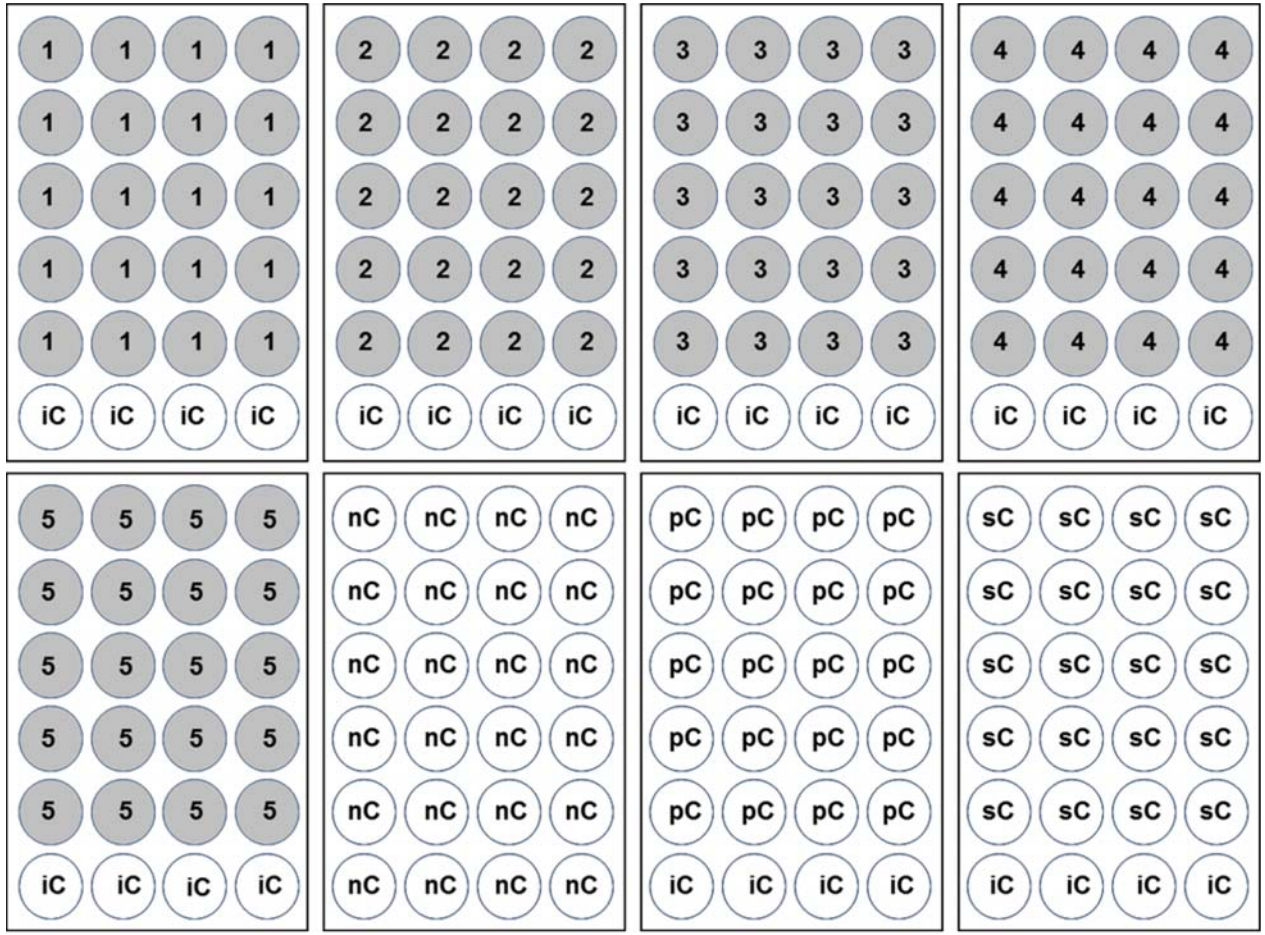


Fig. 3: Normal development of zebrafish (Danio rerio) embryos: (1) 0.75 hrs, 2-cell stage; (2) 1 hr, 4-cell stage; (3) 1.2 hrs, 8-cell stage; (4) 1.5 hrs, 16-cell stage; (5) 4.7 hrs, beginning epiboly; (6) 5.3 hrs, approx. 50 % epiboly (from Braunbeck & Lammer 2006 (40)).

Appendix 4

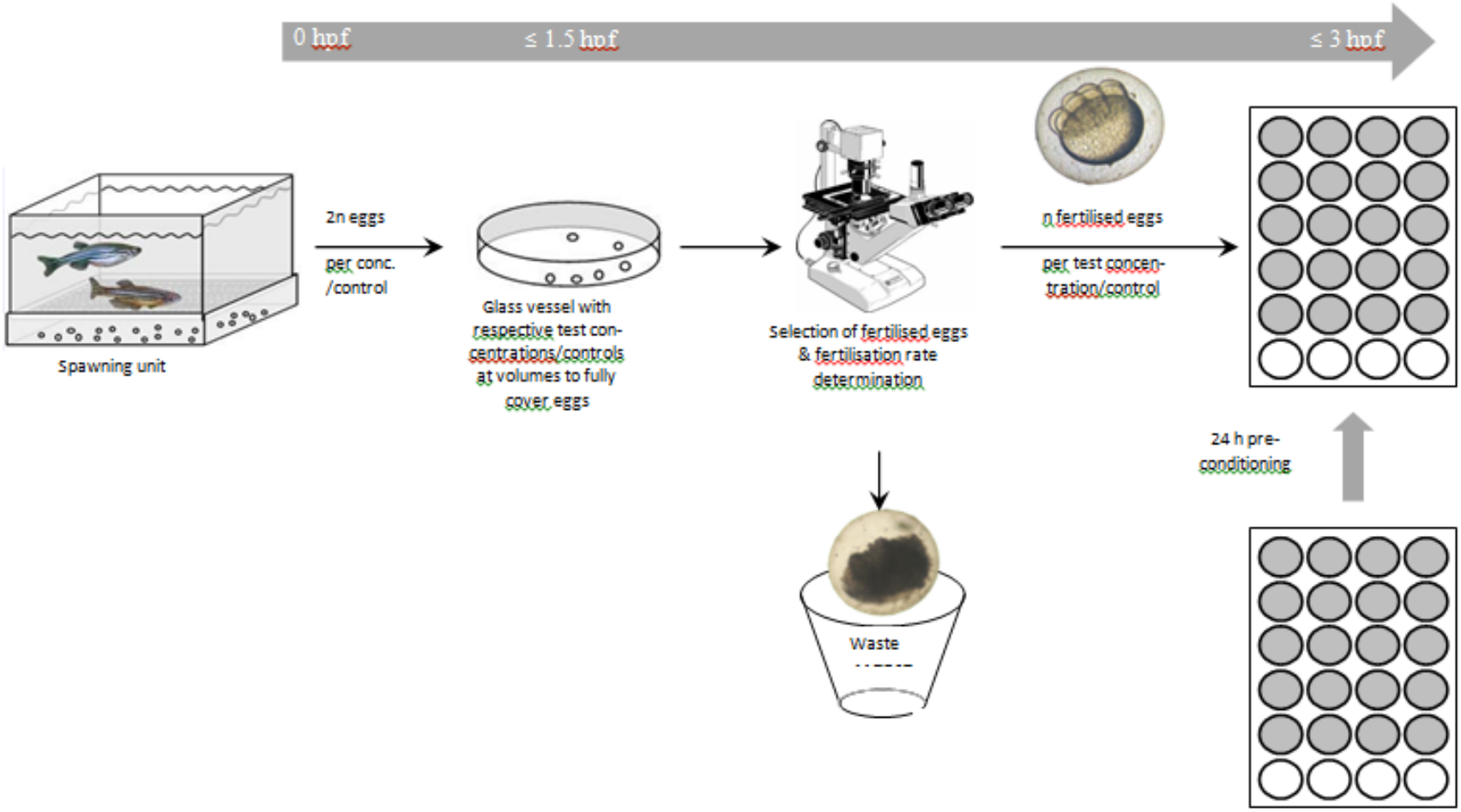
Fig. 1: Layout of 24-well plates



1-5 = five test concentrations / chemical; nC = negative control (dilution water); iC = internal plate control (dilution water);

pC = positive control (3,4-DCA 4mg/l); sC = solvent control

Fig. 2: ***Scheme of the zebrafish embryo acute toxicity test procedure (from left to right):*** *production of eggs, collection of the eggs, pre-exposure immediately after fertilisation in glass vessels, selection of fertilised eggs with an inverted microscope or binocular and distribution of fertilised eggs into 24-well plates prepared with the respective test concentrations/controls, n = number of eggs required per test concentration/control (here 20), hpf = hours post-fertilisation.*



Appendix 5

ATLAS OF LETHAL ENDPOINTS FOR THE ZEBRAFISH EMBRYO ACUTE TOXICITY TEST

The following apical endpoints indicate acute toxicity and, consequently, death of the embryos: *coagulation of the embryo, non-detachment of the tail, lack of somite formation and lack of heartbeat*. The following micrographs have been selected to illustrate these endpoints.

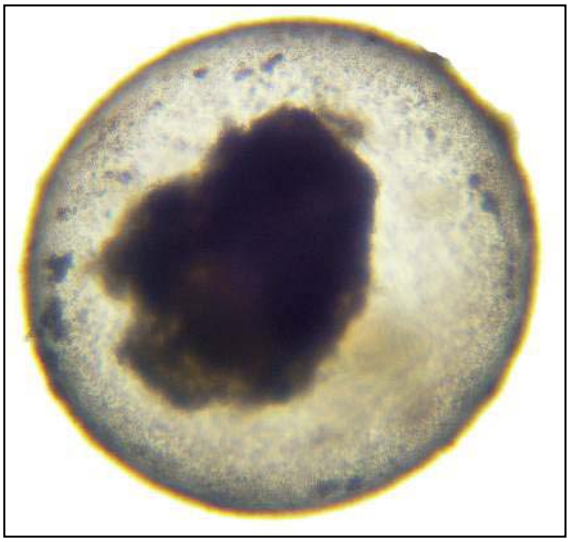


Fig. 1: Coagulation of the embryo: Under bright field illumination, coagulated zebrafish embryos show a variety of intransparent inclusions.

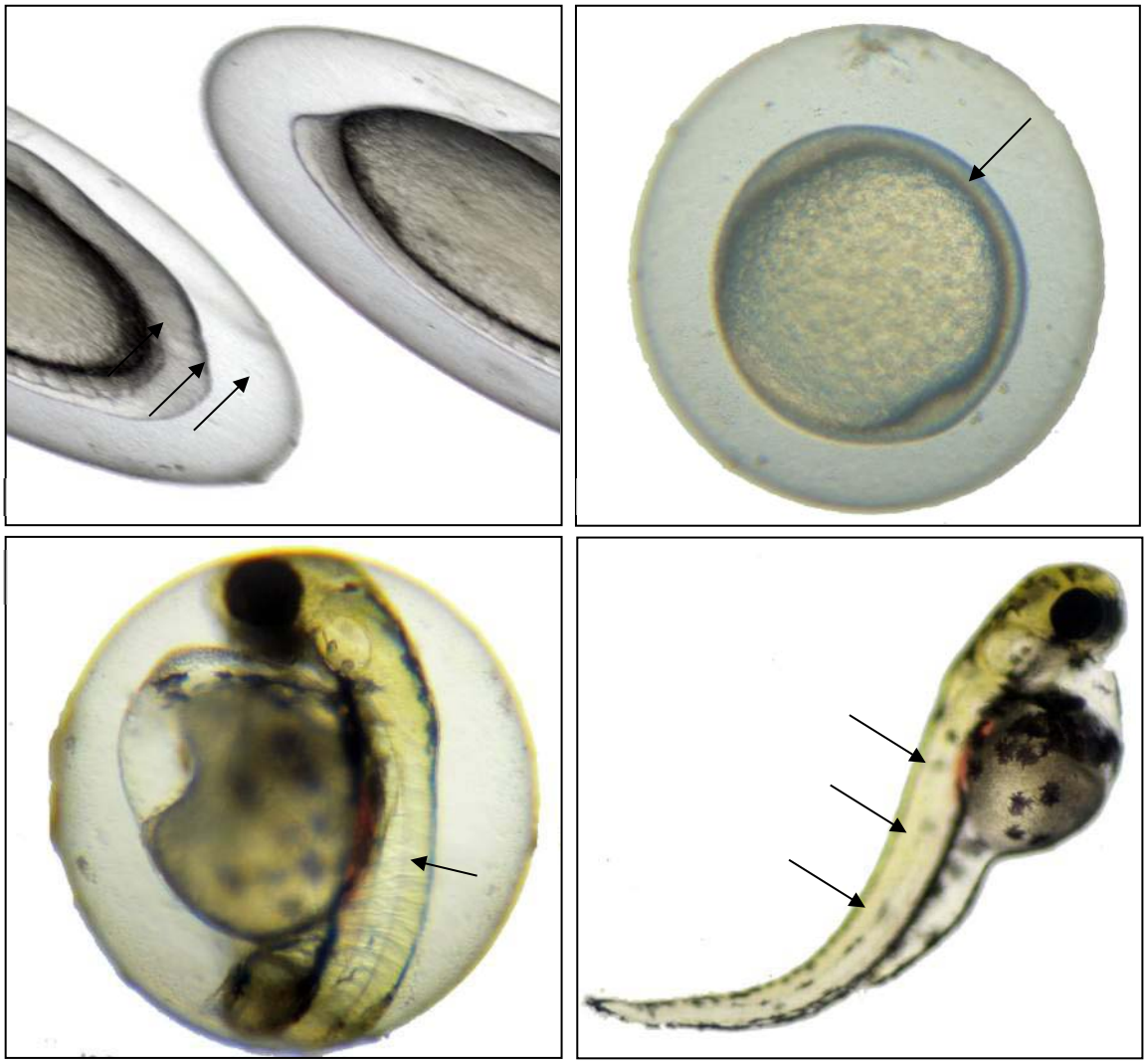


Fig. 2: Lack of somite formation: Although retarded in development by approx. 10 hrs, the 24 hrs old zebrafish embryo in (a) shows well-developed somites (→), whereas the embryo in (b) does not show any sign of somite formation (→). Although showing a pronounced yolk sac oedema (*), the 48 hrs old zebrafish embryo in (c) shows distinct formation of somites (→), whereas the 96 hrs old zebrafish embryo depicted in (d) does not show any sign of somite formation (→). Note also the spinal curvature (scoliosis) and the pericardial oedema (*) in the embryo shown in (d).

Fig. 3: Non-detachment of the tail bud in lateral view (a: →; 96 hrs old zebrafish embryo). Note also the lack of the eye bud (*).

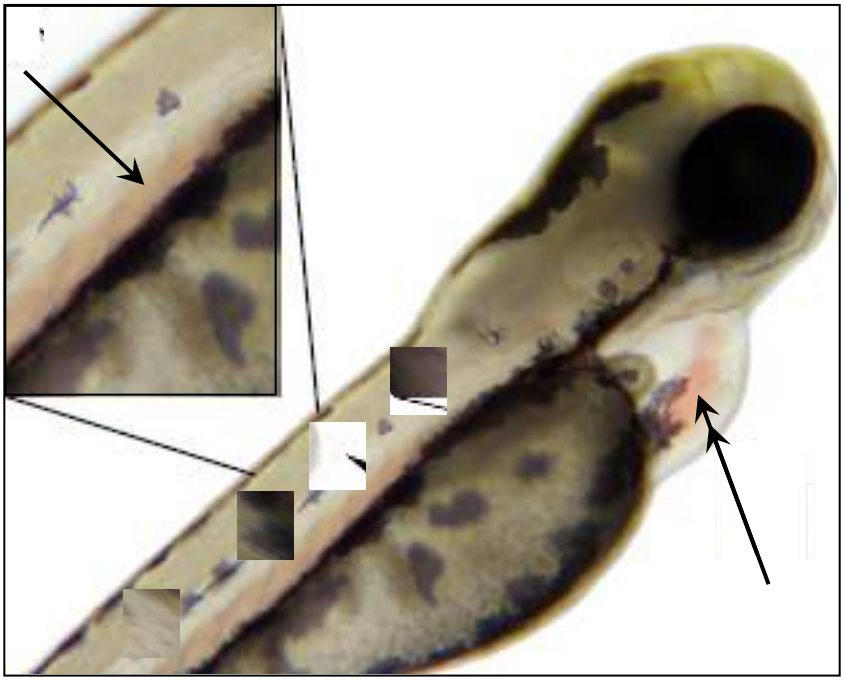


Fig. 4: **Lack of heartbeat** is, by definition, difficult to illustrate in a micrograph. Lack of heartbeat is indicated by non-convulsion of the heart (double arrow). Immobility of blood cells in, e.g. the aorta abdominalis (→ in insert) is not an indicator for lack of heartbeat. Note also the lack of somite formation in this embryo (*, homogenous rather than segmental appearance of muscular tissues). The observation time to record an absence of heartbeat should be at least of one minute with a minimum magnification of 80×.

C.50 Sediment-free *Myriophyllum spicatum* toxicity test

INTRODUCTION

1. This test method is equivalent to OECD test guideline 238 (2014). It is designed to assess the toxicity of chemicals to *Myriophyllum spicatum*, a submersed aquatic dicotyledon, a species of the water milfoils family. It is based on an ASTM existing test method (1) modified as a sediment-free test system (2) to estimate the intrinsic ecotoxicity of test chemicals (independent of the distribution-behaviour of the test chemical between water and sediment). A test system without sediment has a low analytical complexity (only in the water phase) and the results can be analysed in parallel and/or comparison with those obtained in *Lemna sp.* test (3); in addition, the required sterile conditions allow to keep the effects of microorganisms and algae (chemical uptake/ degradation, etc.) as low as possible. This test does not replace other aquatic toxicity tests; it should rather complement them so that a more complete aquatic plant hazard and risk assessment is possible. The test method has been validated by a ring-test (4).

2. Details of testing with renewal (semi-static) and without renewal (static) of the test solution are described. Depending on the objectives of the test and the regulatory requirements, the use of semi-static method is recommended, e.g. for substances that are rapidly lost from solution as a result of volatilisation, adsorption, photodegradation, hydrolysis, precipitation or biodegradation. Further guidance is given in (5). This test method applies to substances, for which the test method has been validated, (see details in the ring-test report (4)) or to formulations, or known mixtures; if a mixture is tested, its constituents should be as far as possible identified and quantified. The sediment-free *Myriophyllum spicatum* test method complements the water-sediment *Myriophyllum spicatum* Toxicity Test (6). Before use of the test method for the testing of a mixture intended for a regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

PRINCIPLE OF THE TEST

3. Continuously growing plant cultures of *Myriophyllum spicatum* (only in modified Andrews' medium, see Appendix 2) are allowed to grow as monocultures in different concentrations of the test chemical over a period of 14 days in a sediment-free test system. The objective of the test is to quantify chemical-related effects on vegetative growth over this period based on assessments of selected measurement variables. Growth of shoot length, of lateral branches and roots as well as development of fresh and dry weight and increase of whorls are the measurement variables. In addition, account is

taken of distinctive qualitative changes in test organisms, such as disfigurement or chlorosis and necrosis indicated by yellowing or white and brown colouring. To quantify chemical-related effects, growth in the test solutions is compared with that of the controls and the concentration bringing about a specified x% inhibition of growth is determined and expressed as the EC_x; "x" can be any value depending on the regulatory requirements, e.g. EC_{10}, EC_{20}, EC_{50}. It should be noted that estimates of EC_{10} and EC_{20} values are only reliable and appropriate in tests where coefficients of variation in control plants fall below the effect level being estimated, i.e. coefficients of variation should be <20% for robust estimation of an EC_{20}.

4. Both average specific growth rate (estimated from assessments of main shoot length and three additional measurement variables) and yield (estimated from the increase in main shoot length and three additional measurement variables) of untreated and treated plants should be determined. Specific growth rate (r) and yield (y) are subsequently used to determine the E_rC_x (e.g. E_rC_{10}, E_rC_{20}, E_rC_{50}) and E_yC_x (e.g. E_yC_{10}, E_yC_{20}, E_yC_{50}), respectively.

5. In addition, the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) may be statistically determined.

INFORMATION ON THE TEST CHEMICAL

6. An analytical method, with adequate sensitivity for quantification of the test chemical in the test medium, should be available. Information on the test chemical which may be useful in establishing the test conditions includes the structural formula, purity and impurities, water solubility, stability in water and light, acid dissociation constant (pK_a), partition coefficient octanol-water (K_{ow}), vapour pressure and biodegradability. Water solubility and vapour pressure can be used to calculate Henry´s Law constant, which will indicate if significant losses of the test chemical during the test period are likely. This will help indicate whether particular steps to control such losses should be taken. Where information on the solubility and stability of the test chemical are uncertain, it is recommended that these be assessed under the conditions of the test, *i.e.* growth medium, temperature, lighting regime to be used in the test.

7. The pH control of the test medium is particularly important, e.g. when testing metals or substances which are hydrolytically unstable. Further guidance for testing chemicals with physical-chemical properties that make them difficult to test is provided in a OECD Guidance Document (5).

VALIDITY OF THE TEST

8. For the test to be valid, the doubling time of main shoot length in the control must be less

than 14 days. Using the media and test conditions described in this test method, this criterion can be attained using a static or semi-static test regime.

9. The mean coefficient of variation for yield based on measurements of shoot fresh weight (i.e. from test initiation to test termination) and the additional measurement variables (see paragraph 37) in the control cultures do not exceed 35% between replicates.

10. More than 50% of the replicates of the control group are kept sterile over the exposure period of 14 days, which means visibly free of contamination by other organisms such as algae, fungi and bacteria (clear solution). *Note*: Guidance on how to assess sterility is provided in the ring-test report (4).

REFERENCE CHEMICAL

11. Reference chemical(s), such as 3,5-dichlorophenol used in the ring test (4), may be tested as a mean of checking the test procedure; from the ring test data, the mean EC_{50}-values of 3,5-DCP for the different response variables (see paragraphs 37-41 of this test method) are between 3.2 mg/l and 6.9 mg/l (see ring test report for details about confidence interval for these values). It is advisable to test a reference chemical at least twice a year or, where testing is carried out at a lower frequency, in parallel to the determination of the toxicity of a test chemical.

DESCRIPTION OF THE METHOD

Apparatus

12. All equipment in contact with the test media should be made of glass or other chemically inert material. Glassware used for culturing and testing purposes should be cleaned of chemical contaminants that might leach into the test medium and should be sterile. The test vessels should be long enough for the shoot in the control vessels to grow in the water phase without reaching the surface of the test medium at the end of the test. Thick-walled borosilicate glass test tubes without lip, inner diameter approximately 20 mm, length approximately 250 mm, with aluminium caps are recommended.

13. Since the modified Andrews' medium contains sucrose (which stimulates the growth of fungi and bacteria), the test solutions have to be prepared under sterile conditions. All liquids as well as equipment are sterilised before use. Sterilisation is carried out via heated air treatment (210 °C) for 4 hours or autoclaving for 20 minutes at 121 °C. In addition, all flasks, dishes, bowls etc. and other equipment undergo flame treatment at a sterile workbench just prior to use.

14. The cultures and test vessels should not be kept together. This is best achieved using separate environmental growth chambers, incubators, or rooms. Illumination and

temperature should be controllable and maintained at a constant level.

Test organism

15. *Myriophyllum spicatum* – a submersed aquatic dicotyledon – is a species of the water milfoils family. Between June and August, inconspicuous pink-white flowers protrude above the water surface. The plants are rooted in the ground by a system of robust rhizomes and can be found in the entire northern hemisphere in eutrophic, however non-polluted and more calciferous still waters with muddy substrate. *Myriophyllum spicatum* prefers fresh water, but is found in brackish water as well.

16. For the sediment-free toxicity test, sterile plants are required. If the testing laboratory does not have regular cultures of *Myriophyllum spicatum*, sterile plant material may be obtained from another laboratory or (unsterile) plant material might be taken from the field or provided by a commercial supplier; if plants come from the field a taxonomic verification of the species should be envisaged. If collected from the field or provided by a commercial supplier, plants should be sterilised (1) and maintained in culture in the same medium as used for testing for a minimum of eight weeks prior to use. Field sites used for collecting starting cultures have to be free of obvious sources of contamination. Great care should be taken to ensure that the correct species is obtained when collecting *Myriophyllum spicatum* from the field, especially in regions where it can hybridise with other *Myriophyllum* species. If obtained from another laboratory they should be similarly maintained for a minimum of three weeks. The source of plant material and the species used for testing should always be reported.

17. The quality and uniformity of the plants used for the test will have a significant influence on the outcome of the test and should therefore be selected with care. Young, rapidly growing plants without visible lesions or discoloration (chlorosis) should be used. Details about preparation of the test organism are given in Appendix 4.

Cultivation

18. To reduce the frequency of culture maintenance (e.g. when no *Myriophyllum* tests are planned for a period), cultures can be held under reduced illumination and temperature (50 $\mu E\ m^{-2}\ s^{-1}$, 20 ± 2 °C). Details of culturing are given in Appendix 3.

19. At least 14 to 21 days before testing, sufficient test organisms are transferred aseptically into fresh sterile medium and cultured for 14 to 21 days under the conditions of the test as a pre culture. Details for preparation of a pre-culture are given in Appendix 4.

Test medium

20. Only one nutrient medium is recommended for *Myriophyllum spicatum* in a sediment-free test system, as described in Appendix 2. A modification of the Andrews' medium is

recommended for culturing and testing with *Myriophyllum spicatum* as described in (1). From five separately prepared nutrient stock solutions with addition of 3% sucrose the modified Andrews' medium will be arranged. Details about preparation of the medium are given in Appendix 2.

21. A tenfold concentrated, modified Andrews' medium is needed for obtaining the test solutions (by dilution as appropriate). The composition of this medium is given in Appendix 2.

Test solutions

22. Test solutions are usually prepared by dilution of a stock solution. Stock solutions of the test chemical are normally prepared by dissolving the chemical in demineralised (*i.e.* distilled or deionised) water. The addition of the nutrients will be achieved by using the tenfold concentrated, modified Andrews' medium.

23. The stock solutions of the test chemical can be sterilised by autoclave at 121 °C for 20 minutes or by sterile filtration, provided that the sterilisation technique used does not denaturise the test chemical. Test solutions can also be prepared in sterile demineralised water or medium, under sterile conditions. The thermo-stability and the adsorption on different surfaces should the taken into account in the selection of the sterilisation procedure of the stock solutions of the test chemical. Because of that, it is recommended that the stock solutions be prepared under sterile conditions, *i.e.* using sterile material for dissolving the test chemical under sterile conditions (e.g. flame sterilisation, laminar-flow hoods, etc.) into sterile water. This technique of preparation of sterile stock solutions is valid for both substances and mixtures.

24. The highest tested concentration of the test chemical should normally not exceed its water solubility under the test conditions. For test chemicals of low water solubility it may be necessary to prepare a concentrated stock solution or dispersion of the chemical using an organic solvent or dispersant in order to facilitate the addition of accurate quantities of the test chemical to the test medium and aid in its dispersion and dissolution. Every effort should be made to avoid the use of such materials. There should be no phytotoxicity resulting from the use of auxiliary solvents or dispersants. For example, commonly used solvents which do not cause phytotoxicity at concentrations up to 100 µl/l, include acetone and dimethylformamide. If a solvent or dispersant is used, its final concentration should be reported and kept to a minimum (≤ 100 µl/l), and all treatments and controls should contain the same concentration of solvent or dispersant. Further guidance on the use of dispersants is given in (5).

Test and control groups

25. Prior knowledge of the toxicity of the test chemical to *Myriophyllum spicatum* from a

range-finding test will help in selecting suitable test concentrations. In the definitive toxicity test, there should normally be five (like in the *Lemna* growth inhibition test, Chapter C.26 of this Annex) to seven test concentrations arranged in a geometric series; they should be chosen in order that the NOEC and EC_{50} values are bracketed by the concentration range (see below). Preferably the separation factor between test concentrations should not exceed 3.2; however, a larger value may be used where the concentration-response curve is flat. Justification should be provided when fewer than five concentrations are used. At least five replicates should be used at each test concentration.

26. In setting the range of test concentrations (for range-finding and/or for the definitive toxicity test), the following should be considered:

To determine an EC_x, test concentrations should bracket the EC_x value to ensure an appropriate level of confidence. For example, if estimating the EC_{50}, the highest test concentration should be greater than the EC_{50} value. If the EC_{50} value lies outside of the range of test concentrations, associated confidence intervals will be large and a proper assessment of the statistical fit of the model may not be possible.

If the aim is to estimate the LOEC/NOEC, the lowest test concentration should be low enough so that growth is not significantly less than that of the control. In addition, the highest test concentration should be high enough so that growth is significantly lower than that in the control. If this is not the case, the test will have to be repeated using a different concentration range (unless the highest concentration is at the limit of solubility or the maximum required limit concentration, e.g. 100 mg/l).

27. Every test should include controls consisting of the same nutrient medium, test organism (choosing plant material as homogeneous as possible, fresh lateral branches from pre-cultures, shortened to 2.5 cm from base), environmental conditions and procedures as the test vessels but without the test chemical. If an auxiliary solvent or dispersant is used, an additional control treatment with the solvent/dispersant present at the same concentration as that in the vessels with the test chemical should be included. The number of replicate control vessels (and solvent vessels, if applicable) should be at least ten.

28. If determination of NOEC is not required, the test design may be altered to increase the number of concentrations and reduce the number of replicates per concentration. However, in any case the number of control replicates should be at least ten.

Exposure

29. Fresh lateral branches from pre-culture shortened to 2.5 cm from base are assigned randomly to the test vessels under aseptic conditions; each test vessel should contain one 2.5 cm lateral branch that should have an apical meristem on one end. The chosen plant material should be the same quality in each test vessel.

30. A randomised design for location of the test vessels in the incubator is required to minimise the influence of spatial differences in light intensity or temperature. A blocked design or random repositioning of the vessels (or repositioning more frequently) when observations are made is also required.

31. If a preliminary stability test shows that the test chemical concentration cannot be maintained (*i.e.* the measured concentration falls below 80% of the measured initial concentration) over the test duration (14 days), a semi-static test regime is recommended. In this case, the plants should be exposed to freshly prepared test and control solutions on at least one occasion during the test (e.g. day 7). The frequency of exposure to fresh medium will depend on the stability of the test chemical; a higher frequency may be needed to maintain near-constant concentrations of highly unstable or volatile chemicals.

32. The exposure scenario through a foliar application (spray) is not covered in this test method.

Test conditions

33. Warm and/or cool white fluorescent lighting should be used to provide light irradiance in the range of about of 100-150 $\mu E\ m^{-2}\ s^{-1}$ when measured as a photosynthetically active radiation (400-700 nm) at points the same distance from the light source as the bottom of the test vessels (equivalent ca. 6000 to 9000 lux) and using a light-dark cycle of 16:8 h. The method of light detection and measurement, in particular the type of sensor, will affect the measured value. Spherical sensors (which respond to light from all angles above and below the plane of measurement) and "cosine" sensors (which respond to light from all angles above the plane of measurement) are preferred to unidirectional sensors, and will give higher readings for a multi-point light source of the type described here.

34. The temperature in the test vessels should be 23 ± 2 °C. Additional care is needed on pH drift in special cases such as when testing unstable chemicals or metals; the pH should remain in a range of 6-9. See (5) for further guidance.

Duration

35. The test is terminated 14 days after the plants are transferred into the test vessels.

Measurements and analytical determinations

36. At the start of the test, the main shoot length of test organism is 2.5 cm (see paragraph 29); it is measured with a ruler (see Appendix 4) or by photography and image analysis. The main shoot length of test organism appearing normal or abnormal needs to be determined at the beginning of the test, at least once during the 14-day exposure period and at test termination. Note: As an alternative for those who do not have image analysis, if the workbench is sterilised prior to addition of plants to test vessels, a sterile

ruler can also be used to measure the length of the main shoot at test initiation and during the test. Changes in plant development, e.g. in deformation in the shoots, appearance, indication of necrosis, chlorosis, break-up or loss of buoyancy and in root length and appearance, should be noted. Significant features of the test medium (e.g. presence of undissolved material, growth of algae, fungi and bacteria in the test vessel) should also be noted.

37. In addition to determinations of main shoot length during the test, effects of the test chemical on three (or more) of the following measurement variables should be also assessed:

    i. Total lateral branches length

    ii. Total shoot length

    iii. Total root length

    iv. Fresh weight

    v. Dry weight

    vi. Number of whorls

    Note 1: The observations made during the range-finding test could help in selecting relevant additional measurements among the six variables listed above.

    Note 2: The determination of the fresh and dry weights (parameters iv and v) is highly desirable.

    Note 3: Due to the fact that sucrose and light (exposure of roots to light during the test) may have an influence on auxin (plant growth hormone) transport carriers, and that some chemicals may have an auxin-type mode of action, the inclusion of root endpoints (parameter iii) is questionable.

    Note 4: The ring test results show high coefficients of variation (> 60%) for the total lateral branch length (parameter i). Total lateral branch length is in any case encompassed within the total shoot length measurement (parameter ii) which shows more acceptable coefficients of variation of < 30%.

    Note 5: Resulting from the above considerations, the recommended main measurement endpoints are: total shoot length, fresh weight and dry weight (parameters ii, iv and v); parameter vi – number of whorls – is left to the experimenter's judgment.

38. Main shoot length and number of whorls have an advantage, in that they can be determined for each test and control vessel at the start, during, and at the end of the test by photography and image analysis, although a (sterile) ruler can also be used.

39. Total lateral branches length, total root length (as a sum of all lateral branches or roots) and total shoot length (as a sum of main shoot length and total lateral branches length)

can be measured with a ruler at the end of exposure.

40. The fresh and/or dry weight should be determined at the start of the test from a sample of the pre-culture representative of what is used to begin the test, and at the end of the test with the plant material from each test and control vessel.

41. Total lateral branches length, total shoot length, total root length, fresh weight, dry weight and number of whorls may be determined as follows:

    i. Total lateral branches length: The lateral branch length may be determined by measuring all lateral branches with a ruler at the end of exposure. The total lateral branches length is the sum of all lateral branches of each test and control vessel.

    ii. Total shoot length: The main shoot length may be determined by image analysis or using a ruler. The total shoot length is the sum of the total lateral branches length and the main shoot length of each test and control vessel at the end of exposure.

    iii. Total root length: The root length may be determined by measuring all roots with a ruler at the end of exposure. The total root length is the sum of all roots of each test and control vessel.

    iv. Fresh weight: The fresh weight may be determined by weighing the test organisms at the end of exposure. All plant material of each test and control vessel will be rinsed with distilled water, dabbed dry with cellulose paper. After this preparation the fresh weight will be determined by weighing. The starting biomass (fresh weight) is determined on the basis of a sample of test organisms taken from the same batch used to inoculate the test vessels.

    v. Dry weight: After the preparations for the determination of the fresh weight the test organisms will be dried at 60 °C to a constant weight. This mass is the dry weight. The starting biomass (dry weight) is determined on the basis of a sample of test organisms taken from the same batch used to inoculate the test vessels.

    vi. Number of whorls: All whorls will be counted out along the main shoot.

Frequency of measurement and analytical determinations

42. If a static test design is used, the pH of each treatment should be measured at the beginning and at the end of the test. If a semi-static test design is used, the pH should be measured in each batch of 'fresh' test solution prior to each renewal and also in the corresponding 'spent' solutions.

43. Light intensity should be measured in the growth chamber, incubator or room at points in the same distance from the light source as the test organisms. Measurements should be made at least once during the test. The temperature of the medium in a surrogate vessel held under the same conditions in the growth chamber, incubator or room should be

recorded at least daily (or continuously with a data logger).

44. During the test, the concentrations of the test chemical(s) are determined at appropriate intervals. In static tests, the minimum requirement is to determine the concentrations at the beginning and at the end of the test.

45. In semi-static tests where the concentrations of the test chemical(s) are not expected to remain within ± 20% of the nominal concentration, it is necessary to analyse all freshly prepared test solutions and the same solutions at each renewal. However, for those tests where the measured initial concentrations of the test chemical(s) are not within ± 20% of nominal but where sufficient evidence can be provided to show that the initial concentrations are repeatable and stable (*i.e.* within the range 80 – 120% of the initial concentration), chemical determinations may be carried out on only the highest and lowest test concentrations. In all cases, determination of test concentrations prior to renewal need only be performed on one replicate vessel at each test concentration (or the contents of the vessels pooled by replicate).

46. If there is evidence that the test concentration has been satisfactorily maintained within ± 20% of the nominal or measured initial concentration throughout the test, analysis of the results can be based on nominal or measured initial values. If the deviation from the nominal or measured initial concentration is not within ± 20 %, analysis of the results should be based on the geometric mean concentration during exposure or models describing the decline of the concentration of the test chemical (5).

Limit test

47. Under some circumstances, e.g. when a preliminary test indicates that the test chemical has no toxic effects at concentrations up to 100 mg/l or up to its limit of solubility in the test medium or in case of a formulation up to its limit of dispersibility, a limit test involving a comparison of responses in a control group and one treatment group (100 mg/l or a concentration equal to the limit of solubility), may be undertaken. It is strongly recommended that this is supported by analysis of the exposure concentration. All previously described test conditions and validity criteria apply to a limit test, with the exception that the number of treatment replicates should be doubled. Growth in the control and treatment group may be analysed using a statistical test to compare means, e.g. a Student's t-test.

DATA AND REPORTING

Response variables

48. The purpose of the test is to determine the effects of a test chemical on the vegetative

growth of *Myriophyllum spicatum*. This test method describes two response variables.

a) Average specific growth rate: This response variable is calculated on the basis of changes in the logarithms of main shoot length, and in addition, on the basis of changes in the logarithms of other measurement parameters, *i.e.* total shoot length, fresh weight, dry weight or number of whorls over time (expressed per day) in the controls and each treatment group. Note: For the measurement parameter total lateral branches length and total root length a calculation of the average specific growth rate is not possible. At the beginning of the test, the test organism has no lateral branches and no roots (based on the preparation from the pre-culture); starting from the value zero, the calculation of the average specific growth rate is not defined.
b) Yield: This response variable is calculated on the basis of changes in main shoot length, and in addition, on the basis of changes in other measurement parameters – *i.e.* preferably total shoot length, fresh weight, dry weight or number of whorls, and other parameters if deemed useful – in the controls and in each treatment group until the end of the test.

49. Toxicity estimates should be based on main shoot length and three additional measurement variables (i.e. preferably total shoot length, fresh weight, dry weight or number of whorls, see paragraph 37 and Notes 2, 4 and 5 to this paragraph), because some chemicals may affect other measurement variables much more than the main shoot length. This effect would not be detected by calculating main shoot length only.

Average specific growth rate

50. The average specific growth rate for a specific period is calculated as the logarithmic increase in the growth variables – main shoot length and three additional measurement variables (*i.e.* total shoot length, fresh weight, dry weight or number of whorls) – using the formula below for each replicate of control and treatments:

$$\mu_{i-j} = \frac{\ln(N_j) - \ln(N_i)}{t}$$

where:

μ_{i-j} : average specific growth rate from time i to j

- N_i : measurement variable in the test or control vessel at time i

N_j : measurement variable in the test or control vessel at time j

t : time period from i to j

For each treatment group and control group, calculate a mean value for growth rate along with variance estimates.

51. The average specific growth rate should be calculated for the entire test period (time "i"

in the above formula is the beginning of the test and time "j" is the end of the test). For each test concentration and control, calculate a mean value for average specific growth rate along with the variance estimates. In addition, the section-by-section growth rate should be assessed in order to evaluate effects of the test chemical occurring during the exposure period (e.g. by inspecting log-transformed growth curves).

52. Percent inhibition of growth rate (I_r) may then be calculated for each test concentration (treatment group) according to the following formula:

$$\%I_r = \frac{(\mu_C - \mu_T)}{\mu_C} \times 100$$

where:

% Ir : percent inhibition in average specific growth rate

μC : mean value for μ in the control

μT : mean value for μ in the treatment group

Yield

53. Effects on yield are determined on the basis of the measurement variable main shoot length and three additional measurement variables (*i.e.* preferably total shoot length, fresh weight, dry weight or number of whorls) present in each test vessel at the start and at the end of the test. For fresh weight or dry weight, the starting biomass is determined on the basis of a sample of test organisms taken from the same batch used to inoculate the test vessels. For each test concentration and control, calculate a mean value for yield along with variance estimates. The mean percent inhibition in yield (% I_y) may be calculated for each treatment group as follows:

$$\%I_y = \frac{(b_C - b_T)}{b_C}$$

where:

% Iy : percent reduction in yield

bC : final biomass minus starting biomass for the control group

bT : final biomass minus starting biomass in the treatment group

Doubling time

54. To determine the doubling time (T_d) of main shoot length and adherence to this validity criterion (see paragraph 8), the following formula is used with data obtained from the control vessels:

$$T_d = \ln 2/\mu$$

Where μ is the average specific growth rate determined as described in paragraphs 50-52.

Plotting concentration-response curves

55. Concentration-response curves relating mean percentage inhibition of the response variable (I_r, or I_y calculated as shown in paragraph 53) and the log concentration of the test chemical should be plotted.

EC_x estimation

56. Estimates of the EC_x should be based upon both average specific growth rate (E_rC_x) and yield (E_yC_x), each of which should in turn be based upon main shoot length, and possibly additional measurement variables (*i.e.* preferably total shoot length, fresh weight, dry weight or number of whorls). This is because there are chemicals that impact main shoot length and other measurement variables differently. The desired toxicity parameters are therefore four EC_x values for each inhibition level x calculated: E_rC_x (main shoot length); E_rC_x (*i.e.* preferably total shoot length, fresh weight, dry weight, or number of whorls); E_yC_x (main shoot length); and E_yC_x (*i.e.* preferably total shoot length, fresh weight, dry weight or number of whorls).

57. It should be noted that EC_x values calculated using these two response variables are not comparable and this difference is recognised when using the results of the test. EC_x values based upon average specific growth rate (E_rC_x) will in most cases be higher than results based upon yield (E_yC_x) – if the test conditions of this test method are adhered to – due to the mathematical basis of the respective approaches. This difference should not be interpreted as a difference in sensitivity between the two response variables, simply the values are different mathematically.

Statistical procedures

58. The aim is to obtain a quantitative concentration-response relationship by regression analysis. It is possible to use a weighted linear regression after having performed a linearising transformation of the response data, for instance with probit or logit or Weibull models (7), but non-linear regression procedures are preferred techniques that better handle unavoidable data irregularities and deviations from smooth distributions. Approaching either zero or total inhibition such irregularities may be magnified by the transformation, interfering with the analysis (7). It should be noted that standard methods of analysis using probit, logit, or Weibull transforms are intended for use on quantal (e.g. mortality or survival) data, and should be modified to accommodate growth rate or yield data. Specific procedures for determination of EC_x values from continuous data can be found in (8) (9) (10).

59. For each response variable to be analysed, use the concentration-response relationship to calculate point estimates of EC_x values. When possible, the 95% confidence limits for each estimate should be determined. Goodness of fit of the response data to the regression model should be assessed either graphically or statistically. Regression analysis should be performed using individual replicate responses, not treatment group means.

60. EC_{50} estimates and confidence limits may also be obtained using linear interpolation with bootstrapping (10), if available regression models/methods are unsuitable for the data.

61. For estimation of the LOEC and hence the NOEC, it is necessary to compare treatment means using analysis of variance (ANOVA) techniques. The mean for each concentration is then compared with the control mean using an appropriate multiple comparison or trend test method. Dunnett's or Williams'test may be useful (12) (13) (14) (15) (16). It is necessary to assess whether the ANOVA assumption of homogeneity of variance holds. This assessment may be performed graphically or by a formal test (15). Suitable tests are Levene's or Bartlett's. Failure to meet the assumption of homogeneity of variances can sometimes be corrected by logarithmic transformation of the data. If heterogeneity of variance is extreme and cannot be corrected by transformation, analysis by methods such as step-down Jonkheere trend tests should be considered. Additional guidance on determining the NOEC can be found in (10).

62. Recent scientific developments have led to a recommendation of abandoning the concept of NOEC and replacing it with regression based point estimates EC_x. An appropriate value for x has not been established for this *Myriophyllum* test. However, a range of 10 to 20% appears to be appropriate (depending on the response variable chosen), and preferably both the EC_{10} and EC_{20} and their confidence limits should be reported.

Reporting

63. The test report includes the following:

Test chemical

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;

- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate).

Multi-constituent substance, UVCBs or mixture:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Test species

- Scientific name and source.

Test conditions

- Test procedure used (static or semi-static).
- Date of start of the test and its duration.
- Test medium.
- Description of the experimental design: test vessels and covers, solution volumes, main shoot length per test vessel at the beginning of the test.
- Test concentrations (nominal and measured as appropriate) and number of replicates per concentration.
- Methods of preparation of stock and test solutions including the use of any solvents or dispersants.
- Temperature during the test.
- Light source, light intensity and homogeneity.
- pH values of the test and control media.
- The method of analysis of test chemical with appropriate quality assessment data (validation studies, standard deviations or confidence limits of analyses).
- Methods for determination of main shoot length and other measurement variables, e.g. total lateral branches length, total shoot length, total root length, fresh weight, dry weight or number of whorls.
- State of the culture (sterile or non-sterile) of each test and control vessel at each observation.
- All deviations from this test method.

Results

- Raw data: main shoot length and other measurement variables in each test and control vessel at each observation and occasion of analysis.

- Means and standard deviations for each measurement variable.

- Growth curves for each measurement variable.

- Calculated response variables for each treatment replicate, with mean values and coefficient of variation for replicates.

- Graphical representation of the concentration/effect relationship.

- Estimates of toxic endpoints for response variables e.g. EC_{50}, EC_{10}, EC_{20}, and associated confidence intervals. If calculated, LOEC and/or NOEC and the statistical methods used for their determination.

- If ANOVA has been used, the size of the effect which can be detected (e.g. the least significant difference).

- Any stimulation of growth found in any treatment.

- Any visual signs of phytotoxicity as well as observations of test solutions.

- Discussion of the results, including any influence on the outcome of the test resulting from deviations from this test method.

LITERATURE

(1) ASTM Designation E 1913-04, Standard Guide for Conducting Static, Axenic, 14-Day Phytotoxicity Tests in Test Tubes with the Submersed Aquatic Macrophyte, *Myriophyllum sibiricum* Komarov.

(2) Maletzki, D. et al. (2010), *Myriophyllum spicatum* als ökotoxikologischer Testorganismus: Methodenentwicklung eines sedimentfreien Testsystems und erste Ergebnisse mit 3,5-Dichlorphenol, *Umweltwiss Schadst Forsch*, No. 22, pp. 702–710.

(3) Chapter C.26 of this Annex: *Lemna* sp. Growth Inhibition Test,

(4) OECD (2014), "*Myriophyllum spicatum* Toxicity Test: Results of an inter-laboratory ring test using a sediment-free test system", OECD Environment, Health and Safety Publications (EHS), Series Testing and Assessment, No. 205, OECD Publishing, Paris.

(5) OECD (2000), "Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures", OECD Environment, Health and Safety Publications (EHS), Series on Testing and Assessment, No. 23, OECD Publishing, Paris.

(6) Chapter C.51 of this Annex: Water-Sediment *Myriophyllum spicatum* Toxicity Test

(7) Christensen, E.R., N. Nyholm (1984), Ecotoxicological Assays with Algae: Weibull Dose-Response Curves, *Environmental Science & Technology*, Vol. 18/9, 713-718.

(8) Nyholm, N. *et al*. (1992), Statistical treatment of data from microbial toxicity tests, *Environmental Toxicology and Chem*istry, Vol. 11/2, pp. 157-167.

(9) Bruce, R.D., D.J. Versteeg (1992), A statistical procedure for modelling continuous toxicity data, *Environmental Toxicology and Chemistry*, Vol. 11/10, pp. 1485-1494.

(10) OECD (2006), "Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application", OECD Environment, Health and Safety Publications (EHS), Series on Testing and Assessment, No. 54, OECD Publishing, Paris.

(11) Norberg-King, T.J. (1988), An interpolation estimate for chronic toxicity: The ICp approach, National Effluent Toxicity Assessment Center Technical Report 05-88, US EPA, Duluth, MN.

(12) Dunnett, C.W. (1955), A multiple comparisons procedure for comparing several treatments with a control, *Journal of the American Statistical Association*, Vol. 50/272, pp. 1096-1121.

(13) Dunnett, C.W. (1964), New tables for multiple comparisons with a control, *Biometrics*, Vol. 20/3, pp. 482-491.

(14) Williams, D.A. (1971), A test for differences between treatment means when several dose levels are compared with a zero dose control, *Biometrics*, Vol. 27/1, pp. 103-117.

(15) Williams, D.A. (1972), The comparison of several dose levels with a zero dose control, *Biometrics*, Vol. 28/2, pp. 519-531.

(16) Brain, P., R. Cousens (1989), An equation to describe dose-responses where there is stimulation of growth at low doses, *Weed Research*, Vol. 29/2, pp. 93-96.

Appendix 1

DEFINITIONS

Biomass is the fresh and/or dry weight of living matter present in a population. In this test the biomass is the sum of main shoot, all lateral branches and all roots.

Chemical is a substance or a mixture.

Chlorosis is the change of the color from green to yellowing of test organism especially of the whorls.

ECx is the concentration of the test chemical dissolved in test medium that results in a x% (e.g. 50%) reduction in growth of *Myriophyllum spicatum* within a stated exposure period (to be mentioned explicitly if deviating from full or normal test duration). To unambiguously denote an EC value deriving from growth rate or yield the symbol "E_rC" is used for growth rate and "E_YC" is used for yield, followed by the measurement variable used, e.g. E_rC (main shoot length).

Growth is an increase in the measurement variable, e.g. main shoot length, total lateral branches length, total shoot length, total root length, fresh weight, dry weight or number of whorls, over the test period.

Growth rate (average specific growth rate) is the logarithmic increase in the measurement variable during the exposure period. *Note*: Growth rate related response variables are independent of the duration of the test as long as the growth pattern of unexposed control organisms is exponential.

Lowest Observed Effect Concentration (LOEC) is the lowest tested concentration at which the chemical is observed to have a statistically significant reducing effect on growth (at $p < 0.05$) when compared with the control, within a given exposure time. 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.

Measurement variables are any type of variables which are measured to express the test endpoint using one or more different response variables. In this test method main shoot

length, total lateral branches length; total shoot length, total root length, fresh weight, dry weight and number of whorls are measurement variables.

Monoculture is a culture with one plant species.

Necrosis is dead (i.e. white or dark brown) tissue of the test organism.

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

Response variable is a variable for the estimation of toxicity derived from any measured variable describing biomass by different methods of calculation. For this test method growth rate and yield are response variables derived from measurement variables like main shoot length, total shoot length, fresh weight, dry weight, or number of whorls.

Semi-static (renewal) test is a test in which the test solution is periodically replaced at specific intervals during the test.

Static test is a test method without renewal of the test solution during the test.

Test chemical is any substance or mixture tested using this test method.

Test endpoint describes the general factor that will be changed relative to control by the test chemical as aim of the test. In this test method the test endpoint is inhibition of growth which may be expressed by different response variables which are based on one or more measurement variables.

Test medium is the complete synthetic growth medium on which test plants grow when exposed to the test chemical. The test chemical will normally be dissolved in the test medium.

UVCB is a substance of unknown or variable composition, complex reaction product or biological material

Yield is value of a measurement variable to express biomass at the end of the exposure period minus the measurement variable at the start of the exposure period. Note: When the growth pattern of unexposed organisms is exponential, yield-based response variables will decrease with the test duration.

Appendix 2

MODIFIED ANDREWS' MEDIUM FOR STOCK CULTURE AND PRE-CULTURE

From five separately prepared nutrient stock solutions the modified Andrews' medium required for stock culture and pre culture will be prepared, with addition of 3 % sucrose.

Table 1: Composition of Andrews' nutrient solution: (ASTM Designation E 1913-04)

Production of nutrient stock solutions			**Production of nutrient solution**
Stock solution	**Chemical**	**Initial weight per 1000 ml**	**ml per 5 l nutrient solution**
1	KCl	74,6 mg	50
	KNO_3	8,08 g	
	$Ca(NO_3)_2$ * 4 H_2O	18,88 g	
2	$MgSO_4$ *7 H_2O	9,86 g	50
3	See below stock solution 3.1		50
4	KH_2PO_4	2,72 g	50
5	$FeSO_4$ * 7 H_2O	0,278 g	50
	Na_2EDTA* 2 H_2O	0,372 g	

Stock solutions can be kept in a refrigerator for 6 months (at 5-10 °C). Only stock solution No. 5 has a reduced shelf life (two months).

Table 2: Production of stock solution 3.1 for preparing stock solution 3

Chemical	Initial weight g/100 ml
$MnSO_4 * 4 H_2O$	0,223
$ZnSO_4 * 7 H_2O$	0,115
H_3BO_3	0,155
$CuSO_4 * 5 H_2O$	0,0125
$(NH_4)_6Mo_7O_{24} * 4 H_2O$	0,0037

After having produced stock solution 3.1 (Table 2), deep-freeze this solution in approximately 11 ml-aliquots (at -18°C at least). The deep-frozen portions have a shelf life of five years.

To produce stock solution 3, defrost stock solution 3.1, fill 10 ml of it into a 1 l volumetric flask and add ultra-pure water up to the flask‘s mark.

To obtain modified Andrews' medium, fill approximately 2500 ml ultra-pure water into a 5 l volumetric flask. After adding 50 ml of each stock solution, fill 90% of the volumetric flask with ultra-pure water and set pH to 5.8.

After this, add 150 g dissolved sucrose (3% per 5 l); then, fill the volumetric flask with ultra-pure water up to the mark. Finally, the nutrient solution is filled into 1 l Schott flasks and autoclaved at 121 °C for 20 minutes.

The nutrient solution thus yielded can be kept sterile in a refrigerator (at 5-10 °C) for three months.

Modified Andrews' medium for Sediment-free toxicity test

From the five nutrient stock solutions already mentioned in Tables 1 and 2, a tenfold concentrated, modified Andrews' medium required for obtaining the test solutions will be prepared, with addition of 30% sucrose. To do so, fill approximately 100 ml ultra-pure water into a 1 l volumetric flask. After adding 100 ml of each of the stock solutions, set pH to 5.8. After this, add 30% dissolved sucrose (300 g per 1000 ml); then, fill the volumetric flask with ultra-pure water up to the mark.

Finally, the nutrient solution is filled into 0.5 l Schott flasks and autoclaved at 121 °C for 20 minutes.

The tenfold concentrated modified nutrient solution thus yielded can be kept sterile in a refrigerator (at 5-10 °C) for three months.

Appendix 3

MAINTENANCE OF STOCK CULTURE

In this Appendix 3 the stock culture of *Myriophyllum spicatum* L[1], a submersed aquatic dicotyledon, a species of the water milfoils family is described. Between June and August, inconspicuous pink-white flowers protrude above the water surface. The plants are rooted in the ground by a system of robust rhizomes and can be found in the entire northern hemisphere in eutrophic, however non-polluted and more calciferous still waters with muddy substrate. *Myriophyllum spicatum* prefers fresh water, but is found in brackish water as well.

For sediment-free stock culture under laboratory conditions, sterile plants are required. Sterile plants are available from the ecotoxicology laboratory of the German Umweltbundesamt (Federal Environment Agency of Germany).

Alternatively, test organisms can be prepared from non-sterile plants in accordance with ASTM designation E 1913-04. See below – extracted from the ASTM Standard Guide – the procedure for culturing *Myriophyllum sibiricum* collected from field:

"If starting from field collected, non-sterile plants, collect *M. sibiricum* turions in the autumn. Place the turions into a 20-l aquarium containing 5 cm of sterile sediment that is covered with silica sand or for example by Turface® and 18 l of reagent water. Aerate the aquarium and maintain at a temperature of 15 °C and a fluence rate of 200 to 300 μmol m^{-2} s^{-1} for 16 h per day. The plant culture in the aquarium may be maintained as a backup source of plants in case the sterile plant cultures are destroyed by mechanical malfunction in the growth cabinet, contamination, or other reason. The plants grown in the aquarium are not sterile and sterile cultures cannot be maintained in a batch culturing system. To sterilize the culture, plants are removed from the aquarium and rinsed under flowing deionized water for about 0.5 h. Under aseptic conditions in a laminar airflow cabinet, the plants are disinfected for less than 20 min (until most of the plant tissue is bleached and just the growing apex is still green) in a 3% (w/v) sodium hypochlorite solution containing 0.01% of a suitable surfactant. Agitate the disinfectant and plant material. Segments with several nodes are transferred into sterile culture tubes containing 45 ml of sterilized modified

[1] Carl von Linné (* May, 23th, 1707 in Råshult /Älmhult; † January, 10th, 1778 in Uppsala).

Andrews' medium and capped with plain culture tube closures. Only one plant segment is placed into each test chamber. Laboratory sealant film is used to secure the closure to the culture vessel. Once a sterile culture has been established, plant segments containing several nodes should be transferred to new test chambers containing fresh liquid nutrient media every ten to twelve days. As demonstrated by culturing on agar plates, the plants must be sterile and remain sterile for eight weeks before testing can be initiated."

Since the modified Andrews' medium contains sucrose (which stimulates the growth of fungi and bacteria), all material, solutions and culturing be conducted under sterile conditions. All liquids as well as equipment are sterilised before use. Sterilisation is carried out via heated air treatment (210 °C) for 4 hours or autoclaving for 20 minutes at 121 °C. In addition, all flasks, dishes, bowls etc and other equipment undergo flame treatment at the sterile workbench just prior to use.

Stock cultures can be maintained under reduced illumination and temperature (50 μE m^{-2} s^{-1}, 20 ± 2 °C) for longer times without needing to be re-established. The *Myriophyllum* growth medium should be the same as that used for testing but other nutrient rich media can be used for stock cultures.

The plant segments are distributed axenically over several 500 ml Erlenmeyer or/and 2000 ml Fernbach flasks, each filled with approximately 450 respectively 1000 ml modified Andrews' medium. Then, the flasks are axenically cellulose plug stoppered.

In addition, thorough flame treatment of equipment at the sterile workbench just prior to use is absolutely necessary. Dependent on number and size, the plants are to be transferred into fresh nutrient solution approximately every three weeks.

Apices as well as segments of the stem middle part for this renewed culture can be used. Number and size of transferred plants (or segments of plants) are dependent on how many plants are needed. For example, you can transfer five shoot segments into one Fernbach flask and three shoot segments into one Erlenmeyer flask, each with a length of 5 cm. Discard any rooted, flowering, dead or otherwise conspicuous parts.

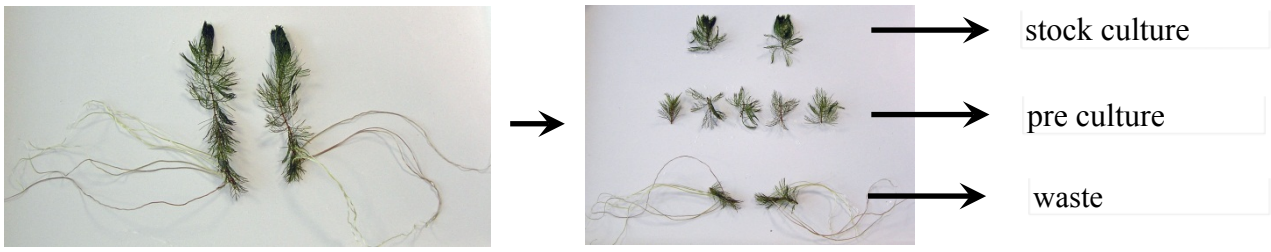


Figure 1: Cutting of plants for the stock and pre culture after 3 weeks of cultivation.

Culturing of plants is to be performed in 500 ml Erlenmeyer and 2000 ml Fernbach flasks in a cooling incubator at 20 ± 2 °C with continuously light at approximately 100-150 µE m^{-2} s^{-1} or 6000-9000 Lux (emitted by chamber illumination with colour temperature "warm white light").

Figure 2: Culturing of plants in a cooling incubator with chamber illumination.

<u>Chemically clean (acid-washed) and sterile glass culture vessels should be used and aseptic handling techniques employed. In the event of contamination of the stock culture e.g. by algae, fungi and/or bacteria a new culture should be prepared or a stock culture from another laboratory should be used to renewal of the one culture.</u>

Appendix 4

MAINTENANCE OF PRE-CULTURE AND PREPARATION OF TEST ORGANISM FOR TESTING

To obtain pre-culture, cut shoots of stock culture into segments with two whorls each; put segments into Fernbach flasks filled with modified Andrews' medium (with 3% sucrose). Each flask can contain up to 50 shoot segments. However, care is to be taken that the segments are vital and do not have any roots and lateral branches or their buds (see figure 1 in Appendix 3).

The pre-culture organisms are cultured for 14 to 21 days under sterile conditions in an environmental chamber with alternating 16/8 hour light/dark phases. Light intensity selected from the range of 100-150 $\mu E\ m^{-2}\ s^{-1}$. The temperature in the test vessels should be 23 ± 2 °C.

Since the modified Andrews' medium contains sucrose (which stimulates the growth of algae, fungi and bacteria), test chemical solutions should be prepared and culturing be conducted under sterile conditions. All liquids as well as equipment are sterilised before use. Sterilisation is carried out via heated air treatment (210 °C) for 4 hours or autoclaving for 20 minutes at 121 °C. In addition, all flasks, dishes, bowls etc. and other equipment undergo flame treatment at the sterile workbench just prior to use.

Shoots are axenically removed from the pre-culture flasks, choosing material that is as homogeneous as possible. Each testing requires at least 60 test organisms (testing with eight test chemical concentrations). For testing, take fresh lateral branches from pre-cultures, shorten them to 2.5 cm from base (measured with ruler) and transfer them into a beaker containing sterile modified Andrews' medium. These fresh lateral branches can be used for the sediment-free *Myriophyllum spicatum* toxicity test.

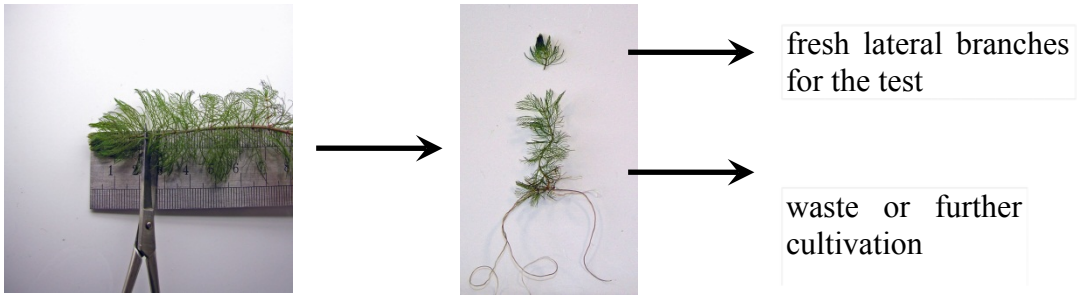


Figure 2: Cutting of plants from the pre culture for the sediment-free *Myriophyllum spicatum* toxicity test.

C.51 water-sediment *Myriophyllum spicatum* toxicity test

INTRODUCTION

1. This test method is equivalent to the OECD test guideline 239 (2014). Test methods are available for the floating, monocotyledonous aquatic plant, *Lemna* species (1) and for algal species (2). These methods are routinely used to generate data to address the risk of test chemicals, in particular chemicals with herbicidal activity, to non-target aquatic plant species. However, in some cases, data for additional macrophyte species may be required. Recent guidance published from the Society of Environmental Toxicology and Chemistry (SETAC) workshop on Aquatic Macrophyte Risk Assessment for Pesticides (AMRAP) proposed that data for a rooted macrophyte species may be required for test chemicals where *Lemna* and algae are known not to be sensitive to the mode of action or if partitioning to sediment is a concern, leading to exposure via root uptake (3). Based on current understanding and experience, *Myriophyllum* spp were selected as the preferred species in cases where data are required for a submerged, rooted dicotyledonous species (4) (5) (6). This test does not replace other aquatic toxicity tests; it should rather complement them so that a more complete aquatic plant hazard and risk assessment is possible. The water-sediment *Myriophyllum spicatum* test method complements the sediment-free *Myriophyllum spicatum* Toxicity Test (7).

2. This document describes a test method, which allows assessment of the effects of a test chemical on the rooted, aquatic plant species *Myriophyllum spicatum*, growing in a water-sediment system. The test method is based partly on existing methods (1) (2) (8) and takes account of recent research related to the risk assessment of aquatic plants (3). The water-sediment method has been validated by an international ring-test conducted with *Myriophyllum* species grown under static conditions, which were exposed to the test chemical through applications made via the water column (9). However, the test system is readily adapted to allow for exposure via spiked sediment or exposure via the water phase in semi-static or pulsed-dose scenarios, although these scenarios have not been formally ring tested. Furthermore, the general method can be used for other rooted, submerged and emergent species including other *Myriophyllum* species (e.g. *Myriophyllum aquaticum*) and *Glyceria maxima* (10). Modifications of test conditions, design and duration may be required for alternative species. In particular, more work is needed to define appropriate procedures for *Myriophyllum aquaticum*. These options are not presented in detail in this test method, which describes the standard approach for exposure of *Myriophyllum spicatum* in a static system via the water phase.

3. This test method applies to substances, for which the test method has been validated, (see details in the ring test report (9)) or to formulations or known mixtures. A *Myriophyllum* test may be conducted to fulfil a Tier 1 data requirement triggered by potential test chemical partitioning to sediment or mode of action/selectivity issues. Equally, a laboratory-based *Myriophyllum* test may be required as part of a higher-tier strategy to address concerns over the risk to aquatic plants. The specific reason for conducting a test will determine the route of exposure (i.e. via water or sediment). Before use of this test method for the testing of a mixture intended for a regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

PRINCIPLE OF THE TEST

4. The test is designed to assess chemical-related effects on the vegetative growth of *Myriophyllum* plants growing in standardised media (water, sediment and nutrients). For this purpose, shoot apices of healthy, non-flowering plants are potted in standardised, artificial sediment, which is supplemented with additional nutrients to ensure adequate plant growth, and then maintained in Smart and Barko medium (Appendix 1). After an establishment period to allow for root formation, plants are exposed to a series of test concentrations added to the water column. Alternatively, exposure via the sediment may be simulated by spiking the artificial sediment with the test chemical and transplanting plants into this spiked sediment. In both cases, plants are subsequently maintained under controlled environmental conditions for 14 days. Effects on growth are determined from quantitative assessments of shoot length, fresh weight and dry weight, as well as qualitative observations of symptoms such as chlorosis, necrosis or growth deformities.

5. To quantify chemical-related effects, growth in the test solutions is compared with the growth of the control plants, and the concentration causing a specified x% inhibition of growth is determined and expressed as the EC_x; "x" can be any value depending on the regulatory requirements, e.g. EC_{10} EC_{20} and EC_{50}. It should be noted that estimates of EC_{10} and EC_{20} values are only reliable and appropriate in tests where coefficients of variation in control plants fall below the effect level being estimated, i.e. coefficients of variation should be <20% for robust estimation of an EC_{20}.

6. Both average specific growth rate (estimated from assessments of shoot length, shoot fresh weight and shoot dry weight) and yield (estimated from the increase in shoot length, shoot fresh weight and shoot dry weight) of untreated and treated plants should be determined. Specific growth rate (r) and yield (y) are subsequently used to determine the E_rC_x (e.g. E_rC_{10}, E_rC_{20}, E_rC_{50}) and E_yC_x (e.g. E_yC_{10}, E_yC_{20}, E_yC_{50}), respectively.

7. If required, the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) may be statistically determined from estimates of average specific growth rates and yield.

INFORMATION ON THE TEST CHEMICAL

8. An analytical method with adequate sensitivity for quantification of the chemicals in the test medium should be available.

9. Information on the test chemical which may be useful in establishing the test conditions includes the structural formula, composition in the case of multi-constituent substances, UVCBs, mixtures or formulations, purity, water solubility, stability in water and light, acid dissociation constant (pK_a), partition coefficient octanol-water (K_{ow}), if available K_d to sediments, vapour pressure and biodegradability. Water solubility and vapour pressure can be used to calculate Henry´s Law constant, which will indicate whether significant losses of the test chemical during the test period are likely. If losses of the test chemicals are likely, the losses should be quantified and the subsequent steps to control such losses should be documented. Where information on the solubility and stability of the test chemical(s) is uncertain, it is recommended that these properties are assessed under the conditions of the test, i.e. growth medium, temperature, lighting regime to be used in the test. *Note*: when light dependent peroxidising herbicides are tested, the laboratory lighting used should contain the equivalent presence of solar ultraviolet light found in natural sunlight.

10. The pH should be measured and adjusted in the test medium as appropriate. The pH control of the test medium is particularly important, e.g. when testing metals or chemicals which are hydrolytically unstable. Further guidance for testing chemicals with physical-chemical properties that make them difficult to test is provided in a OECD Guidance Document (11).

VALIDITY OF THE TEST

11. For the test results to be valid, the mean total shoot length and mean total shoot fresh weight in control plants at least double during the exposure phase of the test. In addition, control plants must not show any visual symptoms of chlorosis and should be visibly free from contamination by other organisms such as algae and/or bacterial films on the plants, at the surface of the sediment and in the test medium.

12. The mean coefficient of variation for yield based on measurements of shoot fresh weight (i.e. from test initiation to test termination) in the control cultures does not exceed 35% between replicates.

REFERENCE CHEMICAL

13. A reference chemical(s), such as 3,5-dichlorophenol used in the ring test (9), should be periodically tested in order to check the performance of the test procedure over time. The ring test data indicate that the mean EC50 values of 3,5-DCP for the different response variables were between 4.7 and 6.1 mg/l (see the ring-test report for details of anticipated confidence interval around these values). It is advisable to test a reference chemical at least twice a year or, where testing is carried out infrequently, in parallel with the definitive toxicity tests. A guide to expected EC50 values for 3,5-DCP is provided in the Statistical Report of the International Ring-test (9).

DESCRIPTION OF THE METHOD

Test apparatus

14. The test should be conducted under controlled environmental conditions, i.e. in a growth chamber, growth room or laboratory, with controllable day length, lighting and temperature (see section "Test conditions", paragraphs 56-58). Stock cultures should be maintained separately from test vessels.

15. The study should be conducted using glass test vessels such as aquaria or beakers; 2-l glass beakers (approximately 24 cm high and 11 cm in diameter) are commonly used. However, other (i.e. larger) vessels may be suitable provided that there is sufficient depth of water to allow unlimited growth and keep the plants submerged throughout the test duration.

16. Plastic or glass plant pots (approximately 9 cm diameter and 8 cm high and 500 ml volume) may be used as containers for potting the plants into the sediment. Alternatively, glass beakers may be used and are preferred in some cases (e.g. testing hydrophobic chemicals or chemicals with high K_{ow}).

17. The choice of pot/beaker size needs to be considered alongside the choice of test vessels and the preferred test design (see below). If using Test Design A (one shoot per pot with three pots per vessel) then smaller pots or larger vessels may be needed. If using Test Design B (three shoots per pot and one pot per vessel) then the stated pot and vessel sizes should be adequate. In all cases, the minimum overlaying water depth should be 12 cm

above the top of the sediment and the ratio of sediment surface area/volume to water surface area/volume should be recorded.

Test organism

18. The general approaches described in this test method can be used to test a range of aquatic plant species. However, the conditions outlined in this test method have been tailored for testing the water milfoil species, *Myriophyllum spicatum*. This species belongs to the dicotyledonous family, Haloragaceae.

19. *Myriophyllum spicatum* (Eurasian water milfoil) is a submerged, rooted species which tolerates a wide range of conditions and is found in both static and flowing water bodies. *M. spicatum* is a perennial which dies back to the roots over winter. Plants usually flower and set seed freely although vegetative propagation from axillary buds or stem fragments that detach naturally or after disturbance, is often the primary method of colonisation.

Cultivation of the test organism

20. Plants may be obtained from natural populations or via aquatic plant suppliers. In both cases, the source of the plants should be documented and species identity should be verified. Great care should be taken to ensure that the correct species is obtained when collecting *Myriophyllum spicatum* from the field, especially in regions where it can hybridise with other *Myriophyllum* species. If in doubt, use of verified laboratory cultures from known sources is recommended. Plants that have been exposed to any chemical contaminants, or collected from sites known to be contaminated, should not be used in this test.

21. In regions where *M. spicatum* is not readily available during the winter months, long-term maintenance of stock cultures may be necessary under glasshouse or laboratory conditions. Stock cultures should be maintained under conditions similar to the test conditions although irradiance and temperature may be reduced in order to reduce the frequency of culture maintenance (e.g. when *Myriophyllum* tests are not planned for a period). Use of larger aquaria and plant pots, than would be used in tests, is recommended in order to allow space for proliferation. Sediment and water-media composition should be the same as would be used for tests although alternative methods of sediment fertilisation may be adopted (e.g. use of commercial slow-release fertiliser formulations)

22. Stock plants should be visibly free of contamination with any other organisms, including snails, filamentous algae, fungi and insects, e.g. eggs or larvae of the moth *Paraponyx stratiotata* and larve or adults of the curculionidae *Eubrychius velutus*. Rinsing plant material in fresh water may be necessary to eliminate visible contamination. In addition,

efforts should be made to minimise the development of unicellular algae and bacterial contamination although complete sterility of the plant material is not necessary. Stock cultures should be monitored and transplanted as necessary to avoid development of algal and bacterial contamination. Aeration of stock cultures may be beneficial should algal or bacterial contamination become problematic.

23. In all cases, plants are cultured/ acclimatised under conditions that are similar, but not necessarily identical, to those used in the test for an adequate period (i.e. > 2 weeks) before their use in a test.

24. Flowering stock cultures should not be used in a test as vegetative growth rates generally decline during and after flowering.

Sediment

25. The following formulated sediment, based on the artificial sediment used in Chapter C.28 of this Annex (8), is recommended for use in this test. The sediment is prepared as described in TM C.28, except for the addition of nutrients as described below:

a) 4-5% peat (dry weight, according to 2 ± 0.5% organic carbon) as close to pH 5.5 to 6.0 as possible; it is important to use peat in powder form, finely ground (preferably particle size < 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% of the particles between 50 and 200 μm).

d) An aqueous nutrient medium is added such that the final sediment batch contains 200 mg/Kg dry sediment of both ammonium chloride and sodium phosphate and the moisture content of the final mixture is in a range of 30-50 %.

e) Calcium carbonate of chemically pure quality ($CaCO_3$) is added to adjust the pH of the final mixture of the sediment to 7.0 ± 0.5.

26. The source of peat, kaolin clay and sand should be known and documented. If the origin is unknown or gives some level of concern, then the respective components should be checked for the absence of chemical contamination (e.g. heavy metals, organochlorine compounds, organophosphorous compounds).

27. The dry constituents of the sediment should be mixed homogenously prior to mixing the aqueous nutrient solution thoroughly into the sediment. The moist sediment should be prepared at least two days before use to allow thorough soaking of the peat and to prevent hydrophobic peat particles floating to the surface when the sediment is overlaid with media; before use, the moist sediment may be stored in the dark.

28. For the test, the sediment is transferred into a suitable size containers, such as plant pots of a diameter which fit into the glass vessels (the sediment surface area should cover approximately 70% or more of the vessel surface area). In cases where the container has holes at the bottom, a piece of filter paper in the bottom of the container will help to keep the sediment within the container. The pots are filled with the sediment such that the sediment surface is level, prior to covering with a thin layer (~ 2 to 3 mm) of an inert material such as sand, fine horticultural grit (or crushed coral) to keep the sediment in place.

Test medium

29. Smart and Barko medium (12) is recommended for culturing and testing *Myriophyllum spicatum*. Preparation of this media is described in the Appendix 1. The pH of the media (water phase) at test initiation should be between 7.5 and 8.0 for optimum plant growth.

Experimental design

30. The test should incorporate a minimum of six replicate test vessels for the untreated control and a minimum of four replicate test vessels for each of a minimum of five concentration levels.

31. If determination of NOEC is not required, the test design may be altered to increase the number of concentrations and reduce the number of replicates per concentration.

32. Each test vessel represents a replicate containing three shoots. There are two options for growing three shoots in each test vessel:

    - Test Design A: one shoot per pot and three pots per vessel.

    - Test Design B: three shoots per pot and one pot per vessel.

    - Alternative test designs of one shoot per pot per test vessel are acceptable provided that replication is adjusted as required to achieve the required validity criteria.

33. The individual test vessels should be randomly assigned to the treatment groups. A randomised design for the location of the test vessels in the test area is required to minimise the influence of spatial differences in light intensity or temperature.

Test chemical concentrations and control groups

34. Concentrations should typically follow a geometric series; the separation factor between test concentrations should not exceed 3.2. Prior knowledge of the toxicity of the test chemical from a range-finding test will help to select suitable test concentrations.

35. To determine an EC_x, test concentrations should bracket the EC_x to ensure an appropriate level of confidence. For example, if estimating the EC_{50}, the highest test concentration should be greater than the EC_{50} value. If the EC_{50} value lies outside of the range of test concentrations, associated confidence intervals will be large and a proper assessment of the statistical fit of the model may not be possible. The use of more test concentrations will improve the confidence interval around the resulting EC_x value.

36. To determine the LOEC/NOEC (optional endpoint), the lowest test concentration should be sufficiently low such that growth is not significantly different from growth in control plants. In addition, the highest test concentration should be sufficiently high such that growth is significantly lower than that in the control. The use of more replicates will enhance the statistical power of the no effect-concentration/ ANOVA design.

Limit test

37. In cases where a range-finding test indicates that the test chemical does not have an adverse effect at concentrations up to 100 mg/l or up to its limit of solubility in the test medium, or in the case of a formulation up to the limit of dispersibility, a limit test may be undertaken to facilitate comparison of responses in a control group and one treatment group – 100 mg/l or a concentration equal to the limit of solubility, or 1000 mg/kg dry sediment. This test should follow the general principles of a standard dose-response test, with the exception that an increase in the minimum number of replicates to six test vessels per control and concentration is advised. Growth in the control and treatment group may be analysed using a statistical test to compare means, e.g. a Student's t-test.

Test solutions

38. Test solutions are usually prepared by dilution of a stock solution, prepared by dissolving or dispersing the test chemical in Smart and Barko media, using demineralised (i.e. distilled or deionised) water (see Appendix 1).

39. The highest test concentration should normally not exceed the water solubility of the test chemical or, in the case of formulations, the dispersibility under the test conditions.

40. For test chemicals of low water solubility, it may be necessary to prepare a concentrated stock solution or dispersion of the chemical using an organic solvent or dispersant in order to facilitate the addition of accurate quantities of the test chemical to the test medium and aid in its dispersion and dissolution. Every effort should be made to avoid the use of such solvents or dispersants. There should be no phytotoxicity resulting from the use of auxiliary solvents or dispersants. For example, commonly used solvents, which do not cause phytotoxicity at concentrations up to 100 µl/l, include acetone and dimethylformamide. If a solvent or dispersant is used, its final concentration should be

reported and kept to a minimum (≤100 µl/l). Under these circumstances all treatments and (solvent) controls should contain the same concentration of solvent or dispersant. Untreated control replicates that do not contain a solvent or dispersant are also incorporated into the test design. Further guidance on the use of dispersants is given in an OECD Guidance Document (11).

TEST PROCEDURE

41. The test procedure varies according to the application route of the test chemical (i.e. via the water or sediment phase). The likely behaviour of the test chemical in a water-sediment system should be considered to inform the choice of exposure regime used in the test (i.e. static or static renewal, spiked water or spiked sediment). Spiked sediment tests may be preferred in some cases for chemicals that are predicted to significantly partition to sediment.

Establishment phase

42. Healthy shoot apices/tips, *i.e.* without side shoots, are cut from the culture plants to give a shoot length of 6 cm (± 1 cm). For Test Design A (one shoot per pot and three pots per vessel) single shoot tips are planted into each pot. For Test Design B (three shoots per pot and one pot per vessel) four to five shoot apices are planted into each pot containing the sediment.

43. In both cases surplus pots should be planted to allow for selection of uniform plants at test initiation, as well as to provide spare plants to be used for inspection of root growth immediately prior to treatment and spare plants to be harvested for shoot biomass and length measurements on Day 0.

44. Shoots are inserted such that approximately three cm, covering at least two nodes, are beneath the sediment surface.

45. Pots are then transferred to test vessels under the same environmental conditions as for the exposure phase and maintained for seven days in Smart and Barko medium to induce root development.

46. After this time, several plants in spare pots should be removed for inspection of root growth. If root growth is not visible (i.e. root tips are not visible), then the establishment phase should be extended until root growth is visible. This step is recommended to ensure that plants are actively growing at the time of test initiation.

Selection of uniform plant material

47. For Test Design A (one shoot per pot and three pots per vessel) pots are selected for uniformity prior to test initiation. For Test Design B (three shoots per pot and one pot per vessel), surplus plants are removed to leave three plants that are uniform in size and appearance.

Exposure via the water phase

48. Pots, selected for uniformity, are placed into the test vessels as required for the experimental design. Smart and Barko medium is then added to the test vessels. Care should be taken to avoid disturbance of the sediment. For this purpose, media may be added using a funnel or a plastic disc to cover the sediment while the medium is poured into the test vessels provided that the disc is removed immediately afterwards. Alternatively, plant pots may be placed in the test vessels after the addition of the media. In both cases, fresh media may be used at the beginning of the exposure phase, if necessary to minimise the potential build-up of algae and bacteria or to allow preparation of single batches of test solution across replicates.

49. The shoot length above sediment is measured, either prior to or after the addition of the medium.

50. The relevant amounts of the test chemical may be added to the test medium before it is added to the test vessels. Alternatively, the test chemical may be introduced into the medium after it has been added to the test vessels. In this case, care should be taken to ensure that the test chemical is homogeneously distributed throughout the test system without disturbing the sediment.

51. In all cases, the appearance (e.g. clear, cloudy, etc.) of the test media is recorded at test initiation.

Exposure via sediment

52. Spiked sediments of the chosen concentration are prepared by addition of a solution of the test chemical directly to fresh sediment. A stock solution of the test chemical dissolved in deionised water is mixed with the formulated sediment by rolling mill, feed mixer or hand mixing. If poorly soluble in water, the test chemical can be dissolved in as small a volume as possible of a suitable organic solvent (e.g. hexane, acetone or chloroform). This solution is then mixed with ca. 10 g of fine quartz sand for one test vessel. The solvent is allowed to evaporate and the sand is then mixed with the suitable amount of sediment per test beaker. Only agents that volatilise readily can be used to solubilise, disperse or emulsify the test chemical. It should be borne in mind that the volume/weight of sand spiked with the test chemical has to be taken into account in the final preparation of the sediment (i.e. the sediment should thus be prepared with less

sand). Care should be taken to ensure that the test chemical added to sediment is thoroughly and evenly distributed within the sediment.

53. The spiked sediment is filled into the pots (as described above). Plants, selected for uniformity and an adequate root system, are removed from the pots used during the establishment phase and transplanted into the spiked sediment as described above.

54. Pots are placed into the test vessels as required for the experimental design. Smart and Barko medium is then added carefully (i.e. using a funnel) in order to avoid disturbance of the sediment. The shoot length above sediment is measured, either prior to or after the addition of the media.

Maintenance of water levels over the test duration

55. The final water volume must be recorded and the water level marked on each test vessel. If water evaporates during the test by more than 10%, the water level should be adjusted with distilled water. If necessary, beakers may be loosely covered by a transparent cover such as transparent plastic lids to minimise evaporation and contamination with algal spores.

Test conditions

56. Warm and/or cool white fluorescent lighting are used to provide light irradiance in the range of about 140 (± 20) $\mu E \cdot m^{-2}$ s^{-1} when measured as a photosynthetically active radiation (400-700 nm) at the water surface and using a light:dark ratio of 16:8 h. Any differences from the selected light irradiance over the test area should not exceed the range of ± 15%.

57. The temperature in the test vessels is 20 ± 2°C.

58. The pH of the control medium should not increase by more than 1.5 units during the test. However, deviation of more than 1.5 units would not invalidate the test when it can be shown that the validity criteria specified previously are met.

Test duration

59. The exposure period is 14 days.

Measurements and analytical determinations

60. After the establishment phase and immediately prior to treatment (i.e. on Day 0), spare plants from five randomly selected pots for the three plants per pot design or 15 pots for

the one plant per pot design, are harvested for assessment of shoot length and fresh and dry weight as described below.

61. For plants transferred into the exposure phase, the following assessments are made as shown in Table 1:

    - Assessments of main shoot length, side shoot number and side shoot length are recorded at least at the end of the exposure period (e.g. on day 14).

    - Visual assessments of plant health are recorded at least three times during the exposure period (e.g. on days 0, 7 and 14).

    - Assessments of shoot fresh weight and dry weight are made at the end of the test (i.e. on Day 14).

62. Shoot length is determined using a ruler. If side shoots are present, their numbers and length should also be measured.

63. Visual assessments of plant health are made by recording the appearance of plants and the general condition of the test medium. Observations to be noted include:

    - Necrosis, chlorosis or other discoloration such as excessive reddening relative to control plants.

    - Development of bacterial or algal contamination;

    - Growth abnormalities such as stunting, altered internodal length, distorted shoots/leaves, the proliferation of side shoots, leaf loss, loss of turgor and stem fragmentation.

    - Visual assessments of root health are made at test termination, by carefully washing sediment from roots to enable observation of the root system. A proposed scale for assessment, relative to control plants, is shown below:

        1) roots absent

        2) few roots

        3) moderate root development

        4) very good root development, similar to controls

64. Assessments of fresh weight are made at the beginning and end of the test by cutting the shoot at sediment level and then blotting dry prior to weighing. Care should be taken to remove sediment particles that may adhere to the base of the shoot. Shoot material is then placed in a drying oven at ca. 60°C and dried to a constant weight, prior to re-weighing and recording the dry weight.

65. A summary of the minimum biological assessments required over the test duration is provided in Table 1.

Table 1: Assessment schedule

Day after treatment (DAT)	***Myriophyllum spicatum***			
	Shoot length, side shoot length and number	**Visual assessment of shoots**	**Shoot fresh and dry weight, Visual assessment of roots**	**pH O_2**
0	A	A	A	A
4	-	-	-	-
7	-	A	-	A
14	A	A	A	A

A : indicates that assessments are required on these occasions
- : indicates that measurements are not required

Frequency of measurements and analytical determinations

66. The temperature of the medium in a supplementary vessel held under the same conditions in the growth chamber, incubator or room should be recorded at least daily (or continuously with a data logger).

67. The pH and dissolved oxygen concentration of the test medium should be checked at test initiation, at least once during the study and at the end of the study in all replicate vessels. On each occasion, measurements should be taken at the same time of the day. If bulk solutions are used to prepare all replicates at each test concentration, then a single measurement of each bulk solution is acceptable on Day 0.

68. Irradiance should be measured in the growth chamber, incubator or room at points equivalent to level of the water surface. Measurements should be made at least once at test initiation or during the test. The method of light detection and measurement, in particular the type of sensor, will affect the measured value. Spherical sensors (which respond to light from all angles above and below the plane of measurement) and "cosine" sensors (which respond to light from all angles above the plane of measurement) are

preferred to unidirectional sensors, and will give higher readings for a multi-point light source of the type described here.

Analytical measurements of test chemical

69. The correct application of the test chemical should be supported by analytical measurements of test chemical concentrations.

70. Water samples should be collected for test chemical analysis shortly after test initiation (i.e. on the day of application for stable test chemicals or one hour after application for chemicals that are not stable) and at test termination for all test concentrations.

71. Concentrations in sediment and sediment pore-water should be determined at test initiation and test termination, at least in the highest test concentration, unless the test chemicals are known to be stable in water (> 80% of nominal). Measurements in sediment and pore-water might not be necessary if the partitioning of the test chemical between water and sediment has been clearly determined in a water/sediment study under comparable conditions (e.g. sediment to water ratio, application method, sediment type).

72. Sampling of sediment at test initiation is likely to disrupt the test system. Hence, additional treated test vessels may be required to facilitate analytical determinations at test initiation and test termination. Similarly, where intermediate assessments are considered necessary, i.e. on day 7, and analyses require large samples of sediment that cannot be easily removed from the test system, analytical determinations should be performed using additional test vessels treated in the same way as those used for biological assessments.

73. Centrifugation at, for example, 10 000 g and 4ºC for 30 minutes is recommended to isolate interstitial water. However, if the test chemical is demonstrated not to absorb to filters, filtration may also be acceptable. In some cases, it might not be possible to analyse concentrations in the pore water if the sample size is too small.

74. In semi-static tests (i.e. exposure via the water phase) where the concentration of the relevant test chemical(s) is not expected to remain within 20% of the nominal concentration over the test duration without renewal of test solutions, used and freshly prepared test solutions should be sampled for analyses of test chemical concentration at each renewal.

75. In cases where the measured initial concentration of the test chemical is not within 20% of nominal but where sufficient evidence can be provided to show that the initial concentrations are repeatable and stable (i.e. within the range of 80-120% of the initial concentration), chemical determinations may be carried out on only the highest and lowest test concentrations.

76. In all cases, determination of test chemical concentrations need only be performed on one replicate vessel at each test concentration. Alternatively, the test solutions of all replicates for each concentration may be pooled for analyses.

77. If there is evidence that the test chemical concentration has been maintained within 20% of the nominal or measured initial concentration throughout the test, then analysis of the results and subsequent derivation of endpoints can be based on nominal or measured initial values.

78. In these cases, effect concentrations should be based on nominal or measured water concentrations at the beginning of the test.

79. However, if there is evidence that the concentration has declined (i.e. is not maintained within 20% of the nominal or measured initial concentration in the treated compartment) throughout the test, then analysis of the results should be based on the geometric mean concentration during exposure or models describing the decline of the concentration of the test chemical in the treated compartment (11).

DATA EVALUATION

80. In cases where use of a solvent / dispersant is required, data from solvent and untreated controls may be pooled for the purposes of statistical analyses provided that the responses of the solvent and untreated controls are not statistically significantly different.

Response variables

81. The purpose of the test is to determine the effects of the test chemical on the vegetative growth of the test species, using two response variables, average specific growth rate and yield, as follows:

Average specific growth rate

82. This response variable is based on changes in the logarithms of total shoot length, total shoot fresh weight and total shoot dry weight, over time in the controls and each treatment group. This variable is calculated for each replicate of each control and treatment group. The mean length and weight of the three plants per test vessel (replicate) and, subsequently, the growth rate for each replicate, should be calculated using the following formula:

$$\mu_{i-j} = \frac{\ln(N_j) - \ln(N_i)}{t}$$

where:
μ_{i-j}: average specific growth rate from time i to j
N_i: measurement variable in the test or control vessel at time i
N_j: measurement variable in the test or control vessel at time j
t: time period from i to j

83. From the replicate responses, a mean value for growth rate along with variance estimates should be calculated for each treatment and control group.

84. The average specific growth rate should be calculated for the entire test period (time "i" in the above formula is the beginning of the test and time "j" is the end of the test). For each test concentration and control, calculate a mean value for average specific growth rate along with the variance estimates.

85. Percent inhibition of growth rate (I_r) may then be calculated for each test concentration (treatment group) according to the following formula:

$$\%I_r = \frac{(\mu_C - \mu_T)}{\mu_C} \times 100$$

where:
% Ir: percent inhibition in average specific growth rate
μC: mean value for μin the control
μT: mean value for μin the treatment group

Yield

86. This response variable is based on changes in total shoot length, total shoot fresh weight and total shoot dry weight, over time in the controls and each treatment group. The mean percent inhibition in yield (% Iy) may be calculated for each treatment group as follows:

$$\%I_y = \frac{(b_C - b_T)}{b_C}$$

where:

% I_y: percent reduction in yield

b_C: final biomass minus starting biomass for the control group

b_T: final biomass minus starting biomass in the treatment group

Plotting concentration-response curves

87. Concentration-response curves relating mean percentage inhibition of the response variable (I_r, or I_y), calculated as shown above and the log concentration of the test chemical should be plotted.

EC_x estimation

88. Estimates of the EC_x (e.g. EC_{50}) should be based upon both average specific growth rate (E_rC_x) and yield (E_yC_x), each of which should in turn be based upon total shoot fresh weight, total shoot dry weight and total shoot length.

89. It should be noted that EC_x values calculated using these two response variables are not comparable and this difference is recognised when using the results of the test. EC_x values based upon average specific growth rate (E_rC_x) will in most cases be higher than results based upon yield (E_yC_x) – if the test conditions of this test method are adhered to – due to the mathematical basis of the respective approaches. This difference should not be interpreted as a difference in sensitivity between the two response variables, simply the values are different mathematically.

Statistical procedures

90. The aim is to obtain a quantitative concentration-response relationship by regression analysis. It is possible to use a weighted linear regression after having performed a linearising transformation of the response data, for instance into probit or logit or Weibull units (13), but non-linear regression procedures are preferred techniques that better handle unavoidable data irregularities and deviations from smooth distributions. Approaching either zero or total inhibition such irregularities may be magnified by the transformation, interfering with the analysis (13). It should be noted that standard methods of analysis using probit, logit, or Weibull transforms are intended for use on quantal (e.g. mortality or survival) data, and should be modified to accommodate growth rate or yield data. Specific procedures for determination of EC_x values from continuous data can be found in (14) (15) (16) (17).

91. For each response variable to be analysed, use the concentration-response relationship to calculate point estimates of EC_x values. The 95% confidence limits for each estimate are determined and goodness of fit of the response data to the regression model should be assessed either graphically or statistically. Regression analysis should be performed using individual replicate responses, not treatment group means.

92. EC_{50} estimates and confidence limits may also be obtained using linear interpolation with bootstrapping (18), if available regression models/methods are unsuitable for the data.

93. For estimation of the LOEC and hence the NOEC, it is necessary to compare treatment means using analysis of variance (ANOVA) techniques. The mean for each concentration is then compared with the control mean using an appropriate test method (e.g. Dunnett's, Williams'tests) (19) (20) (21) (22). It is necessary to assess whether the ANOVA assumption of normal distribution (ND) and variance homogeneity (VH) of variance holds. This assessment should be performed by Shapiro-Wilks-test (ND) or Levene's test (VH). Failure to meet the assumption of ND and homogeneity of variances can sometimes be corrected by logarithmic transformation of the data. If heterogeneity of variance and/or deviation from ND is extreme and cannot be corrected by transformation, analysis by methods such as Bonferroni-Welch-t-test, step-down Jonkheere Terpstra test and Bonferroni-Median-Test should be considered. Additional guidance on determining the NOEC can be found in (16).

REPORTING

94. The test report includes the following details:

Test chemical

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVCBs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Test species

- scientific name and source.

Test conditions

- duration and conditions of establishment phase;
- test procedure used (static, semi-static, pulsed);

- date of start of the test and its duration;

- test medium, *i.e.* sediment and liquid nutrient medium;

  - description of the experimental design: growth chamber/room or laboratory, test vessels and covers, solution volumes, length and weight of test plants per test vessel at the beginning of the test, ratio of sediment surface to water surface, sediment and water volume ratio;

  - test concentrations (nominal and measured as appropriate) and number of replicates per concentration;

  - methods of preparation of stock and test solutions including the use of any solvents or dispersants;

  - temperature during the test;

  - light source, irradiance ($\mu E \cdot m^{-2}\ s^{-1}$)

  - pH values of the test and control media as well as appearance of test media at test initiation and end;

  - oxygen concentrations;

  - the method of analysis with appropriate quality assessment data (validation studies, standard deviations or confidence limits of analyses);

  - methods for determination of measurement variables, e.g., length, dry weight, fresh weight;

  - all deviations from this test method.

Results

  - raw data: shoot length and shoot weight of plants/pot and other measurement variables in each test and control vessel at each observation and occasion of analysis according to the assessment schedule provided in Table 1;

  - means and standard deviations for each measurement variable;

  - growth curves for each concentration;

- doubling time/growth rate in the control based on shoot length and fresh weight including the coefficient of variation for yield of fresh weight;

- calculated response variables for each treatment replicate, with mean values and coefficient of variation for replicates;

- graphical representation of the concentration/effect relationship;

- estimates of toxic endpoints for response variables e.g. EC_{50}, and associated confidence intervals. If calculated, LOEC and/or NOEC and the statistical methods used for their determination;

- if ANOVA has been used, the size of the effect which can be detected (e.g. the minimum significant difference);

- any stimulation of growth found in any treatment;

- any visual signs of phytotoxicity as well as observations of test solutions;

- discussion of the results, including any influence on the outcome of the test resulting from deviations from this test method.

LITERATURE

(1) Chapter C.26 of this Annex: Lemna sp. Growth Inhibition Test.

(2) Chapter C.3 of this Annex: Freshwater Alga and Cyanobacteria, Growth Inhibition Test.

(3) Maltby, L. et al. (2010), Aquatic Macrophyte Risk Assessment for Pesticides, Guidance from the AMRAP Workshop in Wageningen (NL), 14-16 January 2008.

(4) Arts, G.H.P. et al. (2008), Sensitivity of submersed freshwater macrophytes and endpoints in laboratory toxicity tests, Environmental Pollution, Vol. 153, pp. 199-206.

(5) ISO 16191:2013 Water quality – Determination of the toxic effect of sediment on the growth behaviour of Myriophyllum aquaticum.

(6) Knauer, K. et al. (2006), Methods for assessing the toxicity of herbicides to submersed aquatic plants, Pest Management Science, Vol. 62/8, pp. 715-722.

(7) Chapter C.50 of this Annex: Sediment-free *Myriophyllum spicatum* Toxicity Test.

(8) Chapter C.28 of this Annex: Sediment-Water Chironomid Toxicity Using Spiked Water.

(9) Ratte, M., H. Ratte (2014), "Myriophyllum Toxicity Test: Result of a ring test using *M. aquaticum* and *M. spicatum* grown in a water-sediment system", OECD Environment, Health and Safety Publications (EHS), Series on Testing and Assessment, No. 206, OECD Publishing, Paris.

(10) Davies, J. et al. (2003), Herbicide risk assessment for non-target aquatic plants: sulfosulfuron – a case study, Pest Management Science, Vol. 59/2, pp. 231 – 237.

(11) OECD (2000), "Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures", OECD Environment, Health and Safety Publications (EHS), Series on Testing and Assessment, No. 23, OECD Publishing, Paris.

(12) Smart, R.M., J.W. Barko (1985), Laboratory culture of submersed freshwater macrophytes on natural sediments, Aquatic Botany, Vol. 21/3, pp. 251-263.

(13) Christensen, E.R., N. Nyholm (1984), Ecotoxicological Assays with Algae: Weibull Dose-Response Curves, Environmental Science Technology, Vol. 18/9, pp. 713-718.

(14) Nyholm, N. et al. (1992), Statistical treatment of data from microbial toxicity tests, Environmental Toxicology and Chemistry, Vol. 11/2, pp. 157-167.

(15) Bruce, R.D., D.J. Versteeg (1992), A statistical procedure for modelling continuous toxicity data, Environmental Toxicology and Chemistry, Vol. 11/10, 1485-1494.

(16) OECD (2006), "Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application", OECD Environment, Health and Safety Publications (EHS), Series on Testing and Assessment, No. 54, OECD Publishing, Paris.

(17) Brain, P., R. Cousens (1989), An equation to describe dose-responses where there is stimulation of growth at low doses, Weed Research, Vol. 29/2, pp/ 93-96.

(18) Norberg-King, T.J. (1988), An interpolation estimate for chronic toxicity: The ICp approach, National Effluent Toxicity Assessment Center Technical Report 05-88. US EPA, Duluth, MN.

(19) Dunnett, C.W. (1955), A multiple comparisons procedure for comparing several treatments with a control, Journal of the American Statistical Association, Vol. 50/272, pp. 1096-1121.

(20) Dunnett, C.W. (1964), New tables for multiple comparisons with a control, Biometrics, Vol. 20/3, pp. 482-491.

(21) Williams, D.A. (1971), A test for differences between treatment means when several dose levels are compared with a zero dose control, Biometrics, Vol. 27/1, pp. 103-117.

(22) Williams, D.A. (1972), The comparison of several dose levels with a zero dose control, Biometrics, Vol. 28/2, pp. 519-531.

Appendix 1

SMART AND BARKO MEDIUM COMPOSITION

Component	Amount of reagent added to water* (mg/l)
$CaCl_2 \cdot 2\ H_2O$	91.7
$MgSO_4 \cdot 7\ H_2O$	69.0
$NaHCO_3$	58.4
$KHCO_3$	15.4
pH (air equilibrium)	7.9

* demineralised (i.e. distilled or deionised) water

Appendix 2

DEFINITIONS

Biomass is the fresh and/or dry weight of living matter present in a population. In this test the biomass is the sum of main shoot, all lateral branches and all roots.

Chemical is a substance or a mixture.

Chlorosis is the change of the color from green to yellowing of test organism especially of the whorls.

ECx is the concentration of the test chemical dissolved in test medium that results in a x% (e.g. 50%) reduction in growth of *Myriophyllum spicatum* within a stated exposure period (to be mentioned explicitly if deviating from full or normal test duration). To unambiguously denote an EC value deriving from growth rate or yield the symbol "E_rC" is used for growth rate and "$E_\gamma C$" is used for yield, followed by the measurement variable used, e.g. E_rC (main shoot length).

Growth is an increase in the measurement variable, e.g. main shoot length, total lateral branches length, total shoot length, total root length, fresh weight, dry weight or number of whorls, over the test period.

Growth rate (average specific growth rate) is the logarithmic increase in the measurement variable during the exposure period. *Note*: Growth rate related response variables are independent of the duration of the test as long as the growth pattern of unexposed control organisms is exponential.

Lowest Observed Effect Concentration (LOEC) is the lowest tested concentration at which the chemical is observed to have a statistically significant reducing effect on growth (at $p < 0.05$) when compared with the control, within a given exposure time. 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.

Measurement variables are any type of variables which are measured to express the test endpoint using one or more different response variables. In this test method main shoot length, total lateral branches length; total shoot length, total root length, fresh weight, dry weight and number of whorls are measurement variables.

Monoculture is a culture with one plant species.

Necrosis is dead (i.e. white or dark brown) tissue of the test organism.

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

Response variable is a variable for the estimation of toxicity derived from any measured variable describing biomass by different methods of calculation. For this test method growth rate and yield are response variables derived from measurement variables like main shoot length, total shoot length, fresh weight, dry weight, or number of whorls.

Semi-static (renewal) test is a test in which the test solution is periodically replaced at specific intervals during the test.

Static test is a test method without renewal of the test solution during the test.

Test chemical is any substance or mixture tested using this test method.

Test endpoint describes the general factor that will be changed relative to control by the test chemical as aim of the test. In this test method the test endpoint is inhibition of growth which may be expressed by different response variables which are based on one or more measurement variables.

Test medium is the complete synthetic growth medium on which test plants grow when exposed to the test chemical. The test chemical will normally be dissolved in the test medium.

UVCB is a substance of unknown or variable composition, complex reaction product or biological material

Yield is value of a measurement variable to express biomass at the end of the exposure period minus the measurement variable at the start of the exposure period. Note: When the growth pattern of unexposed organisms is exponential, yield-based response variables will decrease with the test duration."