Council of the European Union

Brussels, 22 July 2015
(OR. en)

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ADD 3

MI 490
CHIMIE 57
ENV 483
COMPET 362
ENT 161
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COVER NOTE

From:	European Commission
date of receipt:	10 July 2015
To:	General Secretariat of the Council
Subject:	COMMISSION REGULATION (EU) No …/.. of XXX amending, for the purpose of its adaptation to technical progress, 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) (Text with EEA relevance)

Delegations will find attached document D39048/03.

Encl.: D39048/03

C.38. The Amphibian Metamorphosis Assay

INTRODUCTION

1. This test method is equivalent to OECD test guideline (TG) 231 (2009). The need to develop and validate an assay capable of detecting chemicals active in the thyroid system of vertebrate species originates from concerns that environmental levels of chemicals may cause adverse effects in both humans and wildlife. In 1998, the OECD initiated a high-priority activity to revise existing TGs and to develop new TGs for the screening and testing of potential endocrine disrupters. One element of the activity was to develop a TG for the screening of chemicals active on the thyroid system of vertebrate species. Both an enhancement of the Repeated dose 28-day oral toxicity study in rodents (Chapter B.7 of this Annex) and the Amphibian Metamorphosis Assay (AMA) were proposed. The enhanced test method B.7 underwent validation and a revised test method has been issued. The Amphibian Metamorphosis Assay (AMA) underwent an extensive validation programme which included intra- and inter-laboratory studies demonstrating the relevance and reliability of the assay (1, 2). Subsequently, the validation of the assay was subject to peer-review by a panel of independent experts (3). This test method is the outcome of the experience gained during the validation studies for the detection of thyroid active chemicals, and of work conducted elsewhere in OECD member countries.

PRINCIPLE OF THE TEST

2. The Amphibian Metamorphosis Assay (AMA) is a screening assay intended to empirically identify chemicals which may interfere with the normal function of the hypothalamic-pituitary-thyroid (HPT) axis. The AMA represents a generalised vertebrate model to the extent that it is based on the conserved structures and functions of the HPT axis. It is an important assay because amphibian metamorphosis provides a well-studied, thyroid-dependent process which responds to chemicals active within the HPT axis, and it is the only existing assay that detects thyroid activity in an animal undergoing morphological development.

3. The general experimental design entails exposing stage 51 *Xenopus laevis* tadpoles to a minimum of three different concentrations of a test chemical and a dilution water control for 21 days. There are four replicates of each test treatment. Larval density at test initiation is 20 tadpoles per test tank for all treatment groups. The observational endpoints are hind limb length, snout to vent length (SVL), developmental stage, wet weight, thyroid histology, and daily observations of mortality.

DESCRIPTION OF THE METHOD

Test Species

4. *Xenopus laevis* is routinely cultured in laboratories worldwide and is easily obtainable

through commercial suppliers. Reproduction can be easily induced in this species throughout the year using human chorionic gonadotropin (hCG) injections and the resultant larvae can be routinely reared to selected developmental stages in large numbers to permit the use of stage-specific test protocols. It is preferred that larvae used in the assay are derived from in-house adults. As an alternative although this is not the preferred procedure, eggs or embryos may be shipped to the laboratory performing the test and allowed to acclimate; the shipping of larval stages for use in the test is unacceptable.

Equipment and Supplies

5. The following equipment and supplies are needed for the conduct of this assay:

   a) Exposure system (see description below);

   b) Glass or stainless steel aquaria (see description below);

   c) Breeding tanks;

   d) Temperature controlling apparatus (e.g., heaters or coolers (adjustable to 22º ± 1°C));

   e) Thermometer;

   f) Binocular dissection microscope;

   g) Digital camera with at least 4 megapixel resolution and micro function;

   h) Image digitising software;

   i) Petri dish (e.g. 100 x 15 mm) or transparent plastic chamber of comparable size;

   j) Analytical balance capable of measuring to 3 decimal places (mg);

   k) Dissolved oxygen meter;

   l) pH meter;

   m) Light intensity meter capable of measuring in lux units;

   n) Miscellaneous laboratory glassware and tools;

   o) Adjustable pipettes (10 to 5000 µl) or assorted pipettes of equivalent sizes;

   p) Test chemical in sufficient quantities to conduct the study, preferably of one lot;

   q) Analytical instrumentation appropriate for the chemical on test or contracted analytical services.

Chemical Testability

6. The AMA is based upon an aqueous exposure protocol whereby test chemical is introduced into the test chambers via a flow-through system. Flow-through methods however, introduce constraints on the types of chemicals that can be tested, as determined by the physicochemical properties of the chemical. Therefore, prior to using this protocol, baseline information about the chemical should be obtained that is relevant to determining the testability, and the OECD Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures (4) should be consulted.

Characteristics which indicate that the chemical may be difficult to test in aquatic systems include: high octanol water partitioning coefficients (log K_{ow}), high volatility, susceptibility to hydrolysis, and susceptibility to photolysis under ambient laboratory lighting conditions. Other factors may also be relevant to determining testability and should be determined on a case by case basis. If a successful test is not possible for the chemical using a flow-through test system, a static renewal system may be employed. If neither system is capable of accommodating the test chemical, then the default is to not test it using this protocol.

Exposure System

7. A flow-through diluter system is preferred, when possible, over a static renewal system. If physical and/or chemical properties of any of the test chemicals are not amenable to a flow-through diluter system, then an alternative exposure system (e.g., static-renewal) can be employed. The system components should have water-contact components of glass, stainless steel, and/or Polytetrafluoroethylene. However, suitable plastics can be utilised if they do not compromise the study. Exposure tanks should be glass or stainless steel aquaria, equipped with standpipes that result in an approximate tank volume between 4.0 and 10.0 l and minimum water depth of 10 to 15 cm. The system should be capable of supporting all exposure concentrations and a control, with four replicates per treatment. The flow rate to each tank should be constant in consideration of both the maintenance of biological conditions and chemical exposure (e.g. 25 ml/min). The treatment tanks should be randomly assigned to a position in the exposure system in order to reduce potential positional effects, including slight variations in temperature, light intensity, etc. Fluorescent lighting should be used to provide a photoperiod of 12 hr light: 12 hr dark at an intensity that ranges from 600 to 2000 lux (lumen/m^2) at the water surface. Water temperature should be maintained at 22º ± 1°C, pH maintained between 6.5 to 8.5, and the dissolved oxygen (DO) concentration > 3.5 mg/l (> 40% of the air saturation) in each test tank. As a minimum water temperature, pH and dissolved oxygen should be measured weekly; temperature should preferably be measured continuously in at least one test vessel. Appendix 1 outlines the experimental conditions under which the protocol should be executed. For further information on setting up flow-through exposure systems and/or static renewal systems, please refer to the ASTM Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians (5) and general aquatic toxicology tests.

Water quality

8. Any water that is locally available (e.g. springwater or charcoal-filtered tap water) and permits normal growth and development of *X. laevis* tadpoles could be used. Because local water quality can differ substantially from one area to another, analysis of water quality should be undertaken, particularly, if historical data on the utility of the water for raising *Xenopus* is not available. Special attention should be given that the water is free of copper, chlorine and chloramines, all of which are toxic to frogs and tadpoles. It is further recommended to analyse the water concerning background levels of fluoride, perchlorate and chlorate (by-products of drinking water disinfection) as all of these anions are substrates of the iodine transporter of the thyroid gland and elevated levels of each of these anions may confound the study outcome. Analysis should be

performed before testing begins and the testing water should normally be free from these anions.

Iodide Concentration in Test Water

9. In order for the thyroid gland to synthesise TH, sufficient iodide needs to be available to the larvae through a combination of aqueous and dietary sources. Currently, there are no empirically derived guidelines for minimal iodide concentrations. However, iodide availability may affect the responsiveness of the thyroid system to thyroid active agents and is known to modulate the basal activity of the thyroid gland, an aspect that deserves attention when interpreting the results from thyroid histopathology. Therefore, measured aqueous iodide concentrations from the test water should be reported. Based on the available data from the validation studies, the protocol has been demonstrated to work well when test water iodide (I^-) concentrations ranged between 0.5 and 10 µg/l. Ideally, the minimum iodide concentration in the test water should be 0.5µg/l. If the test water is reconstituted from deionised water, iodine should be added at a minimum concentration of 0.5µg/l. Any additional supplementation of the test water with iodine or other salts should be noted in the report.

Holding of animals

Adult Care and Breeding

10. Adult care and breeding is conducted in accordance with standard guidelines and the reader is directed to the standard guide for performing the Frog Embryo Teratogenesis Assay (FETAX) (6) for more detailed information. Such standard guidelines provide an example of appropriate care and breeding methods, but strict adherence is not required. To induce breeding, pairs (3-5) of adult females and males are injected with human chorionic gonadotropin (hCG). Female and male specimens are injected with approximately 800 IU-1000 IU and 600 IU-800 IU, respectively, of hCG dissolved in 0.6-0.9% saline solution. Breeding pairs are held in large tanks, undisturbed and under static conditions in order to promote amplexus. The bottom of each breeding tank should have a false bottom of stainless steel or plastic mesh which permits the egg masses to fall to the bottom of the tank. Frogs injected in the late afternoon will usually deposit most of their eggs by mid morning of the next day. After a sufficient quantity of eggs are released and fertilised, adults should be removed from the breeding tanks.

Larval Care and Selection

11. After the adults are removed from the breeding tanks, the eggs are collected and evaluated for viability using a representative sub-set of the embryos from all breeding tanks. The best individual spawn(s) (2-3 recommended to evaluate the quality of the spawns) should be retained based upon embryo viability and the presence of an adequate number (minimum of 1500) of embryos. All the organisms used in a study should originate from a single spawning event (i.e., the spawns should not be co-mixed). The embryos are transferred into a large flat pan or dish and all obvious dead or abnormal eggs (see definition in (5)) are removed using a pipette or eyedropper. The sound embryos from each of the three spawns are transferred into three separate

hatching tanks. Four days after being placed in the hatching tanks, the best spawn, based on viability and hatching success, is selected and the larvae are transferred into an appropriate number of rearing tanks at 22° ±1°C. In addition, some additional larvae are moved into extra tanks for use as replacements in the event that mortalities occur in the rearing tanks during the first week. This procedure maintains consistent organism density and thereby reduces developmental divergence within the cohort of a single spawn. All rearing tanks should be siphoned clean daily. As a precaution, vinyl or nitrile gloves are preferred to latex gloves. Mortalities should be removed daily and replacement larvae should be added back to maintain the organism density during the first week. Feeding should occur at least twice per day.

12. During the pre-exposure phase, tadpoles are acclimated to the conditions of the actual exposure phase, including the type of food, temperature, light-dark cycle and the culture medium. Therefore, it is recommended that the same culture/dilution water be used during the pre-exposure phase and the exposure phase. If a static culture system is used for maintaining tadpoles during the pre-exposure phase, the culture medium should be replaced completely at least twice per week. Crowding, caused by high larval densities during the pre-exposure period, should be avoided because such effects could markedly affect tadpole development during the subsequent testing phase. Therefore, the rearing density should not exceed approximately four tadpoles/l culture medium (static exposure system) or 10 tadpoles/l culture medium (with e.g. 50 ml/min flow rate in the pre-exposure or culturing system). Under these conditions, tadpoles should develop from stages 45/46 to stage 51 within twelve days. Representative tadpoles of this stock population should be inspected daily for developmental stage in order to estimate the appropriate time point for initiation of exposure. Care should be used to minimise stress and trauma to the tadpoles, especially during movement, cleaning of aquaria, and manipulation of larvae. Stressful conditions/activities should be avoided such as loud and/or incessant noise, tapping on aquaria, vibrations in the aquaria, excessive activity in the laboratory, and rapid changes in environmental media (light availability, temperature, pH, DO, water flow rates, etc.) If tadpoles do not develop to stage 51 within 17 days after fertilisation, excessive stress should be considered as a potential culprit.

Larval Culture and Feeding

13. Tadpoles are fed with e.g. the commercial tadpole feed used in the validation studies (see also appendix 1) throughout the pre-exposure period (after Nieuwkoop and Faber (NF) stage 45/46 (8)) and during the entire test period of 21 days, or other diet that has demonstrated to allow equal performance of the Amphibian Metamorphosis Assay. The feeding regime during the pre-exposure period should be carefully adjusted to meet the demands of the developing tadpoles. That is, small portions of food should be provided to the newly hatched tadpoles several times per day (at least twice). Excess food should be avoided in order *i)* to maintain water quality and *ii)* to prevent the clogging of gill filters with food particles and detritus. For the tadpole feed used in the validation studies, the daily food rations should be increased along with tadpole growth to approximately 30 mg/animal/day shortly before test initiation. This commercially available feed has been shown in the validation studies to support proper growth and development of *X. laevis* tadpoles, and is a fine particulate that stays

suspended in the water column for a long period of time and is subject to washing out with the flow. Therefore, the total daily amount of food should be divided into smaller portions and fed at least twice daily. For this feed the feeding regime is outlined in Table 1. Feeding rates should be recorded. It can be fed dry or as a stock solution prepared in dilution water. Such a stock solution should be freshly prepared every other day and stored at 4º C when not in use.

Table 1. Feeding regime with commercial tadpole feed used in the validation studies for *X. laevis* tadpoles during the in-life portion of the AMA in flow-through conditions

Study Day	Food ration (mg feed/animal/day)
0-4	30
5-7	40
8-10	50
11-14	70
15-21	80

Analytical Chemistry

14. Prior to conducting a study, the stability of the test chemical should be evaluated using existing information on its solubility, degradability and volatility. Test solutions from each replicate tank at each concentration should be sampled for analytical chemistry analyses at test initiation (day 0), and weekly during the test for a minimum of four samples. It is also recommended that each test concentration be analysed during system preparation, prior to test initiation, to verify system performance. In addition, it is recommended that stock solutions be analysed when they are changed, especially if the volume of the stock solution does not provide adequate amounts of chemical to span the duration of routine sampling periods. In the case of chemicals which cannot be detected at some or all of the concentrations used in a test, stock solutions should be measured and system flow rates recorded in order to calculate nominal concentrations.

Chemical Delivery

15. The method used to introduce the test chemical to the system can vary depending on its physicochemical properties. Water soluble chemicals can be dissolved in aliquots of test water at a concentration which allows delivery at the target test concentration in a flow-through system. Chemicals which are liquid at room temperature and sparingly soluble in water can be introduced using liquid:liquid saturator methods. Chemicals which are solid at room temperature and are sparingly soluble in water can be introduced using glass wool column saturators (7). The preference is to use a carrier-free test system, however different test chemicals will possess varied physicochemical properties that will likely require different approaches for preparation of chemical exposure water. It is preferred that effort be made to avoid solvents or carriers because: *i)* certain solvents themselves may result in toxicity and/or undesirable or

unexpected endocrinological responses, *ii)* testing chemicals above their water solubility (as can frequently occur through the use of solvents) can result in inaccurate determinations of effective concentrations, and *iii)* the use of solvents in longer-term tests can result in a significant degree of "biofilming" associated with microbial activity. For difficult to test chemicals, a solvent may be employed as a last resort, and the OECD Guidance Document on aquatic toxicity testing of difficult substances and mixtures should be consulted (4) to determine the best method. The choice of solvent will be determined by the chemical properties of the chemical. Solvents which have been found to be effective for aquatic toxicity testing include acetone, ethanol, methanol, dimethyl formamide and triethylene glycol. In case a solvent carrier is used, solvent concentrations should be below the chronic No Observed Effect Concentration (NOEC); the OECD Guidance Document recommends a maximum of 100µl/l; a recent review recommends that solvent concentrations as low as 20µl/l of dilution water be used (12). If solvent carriers are used, appropriate solvent controls should be evaluated in addition to non-solvent controls (clean water). If it is not possible to administer a chemical via the water, either because of physicochemical characteristics (low solubility) or limited chemical availability, introducing it via the diet may be considered. Preliminary work has been conducted on dietary exposures; however, this route of exposure is not commonly used. The choice of method should be documented and analytically verified.

Selection of test concentrations

Establishing the High Test Concentration

16. For the purposes of this test, the high test concentration should be set by the solubility limit of the test chemical; the maximum tolerated concentration (MTC) for acutely toxic chemicals; or 100 mg/l, whichever is lowest.

17. The MTC is defined as the highest test concentration of the chemical which results in less than 10% acute mortality. Using this approach assumes that there are existing empirical acute mortality data from which the MTC can be estimated. Estimating the MTC can be inexact and typically requires some professional judgment. Although the use of regression models may be the most technically sound approach to estimating the MTC, a useful approximation of the MTC can be derived from existing acute data by using 1/3 of the acute LC_{50} value. However, acute toxicity data may be lacking for the species on test. If species specific acute toxicity data are not available, then a 96-hour LC_{50} test can be completed with tadpoles that are representative (i.e., same stage) of those on test in the AMA. Optionally, if data from other aquatic species are available (e.g. LC_{50} studies in fish or other amphibian species), then professional judgment may be used to estimate a likely MTC based on inter-species extrapolation.

18. Alternatively, if the chemical is not acutely toxic and is soluble above 100 mg/l, then 100 mg/l should be considered the highest test concentration (HTC), as this concentration is typically considered "practically non-toxic."

19. Although not the recommended procedure, static renewal methods may be used where flow-through methods are inadequate to achieve the MTC. If static renewal methods

are used, then the stability of the test chemical concentration should be documented and remain within the performance criteria limits. Twenty-four hour renewal periods are recommended. Renewal periods exceeding 72 hours are not acceptable. Additionally, water quality parameters (e.g. DO, temperature, pH, etc.) should be measured at the end of each renewal period, immediately prior to renewal.

Test Concentration Range

20. There is a required *minimum* of three test concentrations and a clean water control (and vehicle control if necessary). The minimum test concentration differential between the highest and lowest should be about one order of magnitude. The maximum dose separation is 0.1 and the minimum is 0.33.

PROCEDURE

Test Initiation and Conduct

Day 0

21. The exposure should be initiated when a sufficient number of tadpoles in the pre-exposure stock population have reached developmental stage 51, according to Nieuwkoop and Faber (8), and which are less than or equal to 17 days of age post fertilisation. For selection of test animals, healthy and normal looking tadpoles of the stock population should be pooled in a single vessel containing an appropriate volume of dilution water. For developmental stage determination, tadpoles should be individually removed from the pooling tank using a small net or strainer and transferred to a transparent measurement chamber (e.g. 100 mm Petri dish) containing dilution water. For stage determination, it is preferred not to use anaesthesia, however one may individually anaesthetise the tadpoles using 100 mg/l tricaine methanesulfonate (e.g. MS-222), appropriately buffered with sodium bicarbonate (pH 7.0), before handling. If used, methodology for appropriately using e.g. MS-222 for anaesthesia should be obtained from experienced laboratories and reported with the test results. Animals should be carefully handled during this transfer in order to minimise handling stress and to avoid any injury.

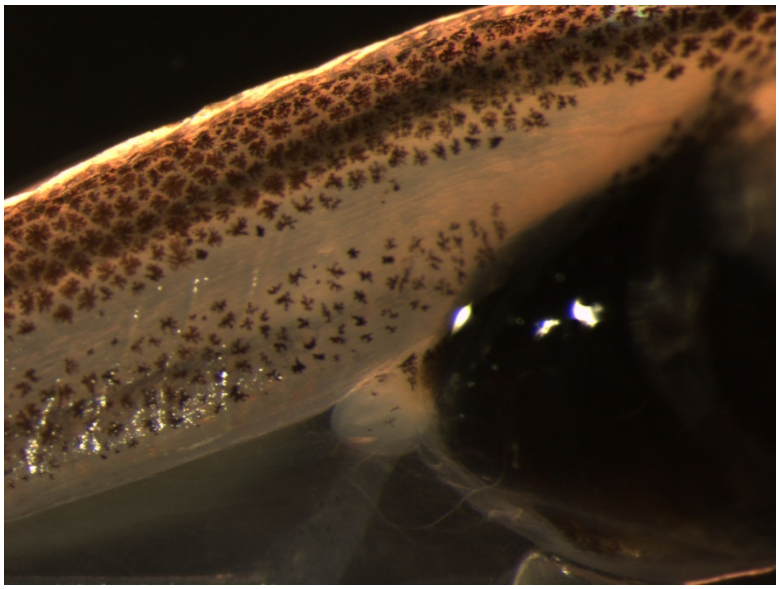
22. The developmental stage of the animals is determined using a binocular dissection microscope. To reduce the ultimate variability in developmental stage, it is important that this staging be conducted as accurately as possible. According to Nieuwkoop and Faber (8), the primary developmental landmark for selecting stage 51 organisms is hind limb morphology. The morphological characteristics of the hind limbs should be examined under the microscope. While the complete Nieuwkoop and Faber (8) guide should be consulted for comprehensive information on staging tadpoles, one can reliably determine stage using prominent morphological landmarks. The following table can be used to simplify and standardise the staging process throughout the study by identifying those prominent morphological landmarks associated with different stages, assuming that development is normal.

Table 2. Prominent morphological staging landmarks based on Neuwkoop and Faber guidance.

Prominent Morphological Landmarks	Developmental Stage															
	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66
Hindlimb	X	X	X	X	X	X	X									
Forelimb						X	X	X	X	X						
Craniofacial structure										X	X	X	X			
Olfactory nerve morphology											X	X	X			
Tail length													X	X	X	X

23. For test initiation, all tadpoles should be at stage 51. The most prominent morphological staging landmark for that stage is hind limb morphology, which is demonstrated in Figure 1.

Figure 1. Hind limb morphology of a stage 51 X. laevis tadpole.



24. In addition to the developmental stage selection, an optional size selection of the experimental animals may be used. For this purpose, the whole body length (not SVL) should be measured at day 0 for a sub-sample of approximately 20 NF stage 51 tadpoles. After calculation of the mean whole body length for this group of animals,

minimum and maximum limits for the whole body length of experimental animals can be set by allowing a range of the mean value ± 3 mm (mean values of whole body length range between 24.0 and 28.1 mm for stage 51 tadpoles). However, developmental staging is the primary parameter in determining the readiness of each test animal. Tadpoles exhibiting grossly visible malformations or injuries should be excluded from the assay.

25. Tadpoles that meet the stage criteria described above are held in a tank of clean culture water until the staging process is completed. Once the staging is completed, the larvae are randomly distributed to exposure treatment tanks until each tank contains 20 larvae. Each treatment tank is then inspected for animals with abnormal appearance (e.g., injuries, abnormal swimming behaviour, etc.). Overtly unhealthy looking tadpoles should be removed from the treatment tanks and replaced with larvae newly selected from the pooling tank.

Observations

26. For more in-depth information on test termination procedures and processing of tadpoles, refer to the OECD Guidance Document on Amphibian Thyroid Histology (9).

Day 7 Measurements

27. On day 7, five randomly chosen tadpoles per replicate are removed from each test tank. The random procedure used should give each organism on test equal probability of being selected. This can be achieved by using any randomising method but requires that each tadpole be netted. Tadpoles not selected are returned to the tank of origin and the selected tadpoles are humanely euthanised in 150 to 200 mg/l e.g. MS-222, appropriately buffered with sodium bicarbonate to achieve pH 7.0. The euthanised tadpoles are rinsed in water and blotted dry, followed by body weight determination to the nearest milligram. Hind limb length, snout to vent length, and developmental stage (using a binocular dissection microscope) are determined for each tadpole.

Day 21 Measurements (Test Termination)

28. At test termination (day 21), the remaining tadpoles are removed from the test tanks and humanely euthanised in 150 to 200 mg/l e.g. MS-222, appropriately buffered with sodium bicarbonate, as above. Tadpoles are rinsed in water and blotted dry, followed by body weight determination to the nearest milligram. Developmental stage, SVL, and hind limb lengths are measured for each tadpole.

29. All larvae are placed in Davidson's fixative for 48 to 72 hours either as whole body samples or as trimmed head tissue samples containing the lower jaw for histological assessments. For histopathology, a total of five tadpoles should be sampled from each replicate tank. Since follicular cell height is stage dependent (10), the most appropriate sampling approach for histological analyses is to use stage-matched individuals, whenever possible. In order to select stage-matched individuals, all larvae should first be staged prior to selection and subsequent processing for data collection and

preservation. This is necessary because normal divergence in development will result in differential stage distributions within each replicate tank.

30. Animals selected for histopathology (n=5 from each replicate) should be matched to the median stage of the controls (pooled replicates) whenever possible. If there are replicate tanks with more than five larvae at the appropriate stage, then five larvae are randomly selected.

31. If there are replicate tanks with less than five larvae at the appropriate stage, then randomly selected individuals from the next lower or upper developmental stage should be sampled to reach a total sample size of five larvae per replicate. Preferably, the decision to sample additional larvae from either the next lower or upper developmental stage should be made based on an overall evaluation of the stage distribution in the control and chemical treatments. That is, if the chemical treatment is associated with a retardation of development, then additional larvae should be sampled from the next lower stage. In turn, if the chemical treatment is associated with an acceleration of development, then additional larvae should be sampled from the next upper stage.

32. In cases of severe alterations of tadpole development due to treatment with a test chemical, there might be no overlap of the stage distribution in the chemical treatments with the calculated control median developmental stage. In only these cases, the selection process should be modified by using a stage different from the control median stage to achieve a stage-matched sampling of larvae for thyroid histopathology. Furthermore, if stages are indeterminate (i.e., asynchrony), then 5 tadpoles from each replicate should be randomly chosen for histological analysis. The rationale underlying sampling of any larvae that are not at a stage equivalent to the control median developmental stage should be reported.

Determination of Biological Endpoints

33. During the 21 day exposure phase, measurement of primary endpoints is performed on days 7 and 21, however daily observation of test animals is necessary. Table 3 provides an overview of the measurement endpoints and the corresponding observation time points. More detailed information for technical procedures for measurement of apical endpoints and histological assessments is available in the OECD guidance documents (9).

Table 3. Observation time points for primary endpoints in the AMA.

Apical Endpoints	Daily	Day 7	Day 21
-Mortality	●		
-Developmental Stage		●	●
-Hind Limb Length		●	●
-Snout-Vent Length		●	●
-Wet Body Weight		●	●
-Thyroid Gland Histology			●

Apical Endpoints

34. Developmental stage, hind limb length, SVL and wet weight are the apical endpoints of the AMA, and each is briefly discussed below. Further technical information for collecting these data is available in the guidance documents referenced including procedures for computer-assisted analysis which are recommended for use.

Developmental Stage

35. The developmental stage of *X. laevis* tadpoles is determined using the staging criteria of Nieuwkoop and Faber (8). Developmental stage data are used to determine if development is accelerated, asynchronous, delayed or unaffected. Acceleration or delay of development is determined by making a comparison between the median stage achieved by the control and treated groups. Asynchronous development is reported when the tissues examined are not malformed or abnormal, but the relative timing of the morphogenesis or development of different tissues is disrupted within a single tadpole.

Hind Limb Length

36. Differentiation and growth of the hind limbs are under control of thyroid hormones and are major developmental landmarks already used in the determination of developmental stage. Hind limb development is used qualitatively in the determination of developmental stage, but is considered here as a quantitative endpoint. Therefore, hind limb length is measured as an endpoint to detect effects on the thyroid axis (Figure 2). For consistency, hind limb length is measured on the left hind limb. Hind limb length is evaluated both at day 7 and at day 21 of the test. On day 7, measuring hind limb length is straightforward, as illustrated in Figure 2. However, measuring hind limb length on day 21 is more complicated due to bends in the limb. Therefore, measurements of hind limb length at day 21 should originate at the body wall and follow the midline of the limb through any angular deviations. Changes in hind limb length at day 7, even if not evident at day 21, are still considered significant for potential thyroid activity. Length measurements are acquired from digital photographs using image analysis software as described in the OECD Guidance Document on Amphibian Thyroid Histology (9).

Body Length and Wet Weight

37. Determinations of snout to vent length (SVL) (Figure 2) and wet weight are included in the test protocol to assess possible effects of test chemicals on the growth rate of tadpoles in comparison to the control group and are useful in detecting generalised toxicity to the test chemical. Because the removal of adherent water for weight determinations can cause stressful conditions for tadpoles and may cause skin damage, these measurements are performed on the day 7 sub-sampled tadpoles and all remaining tadpoles at test termination (day 21). For consistency, use the cranial aspect of the vent as the caudal limit of the measurement.

38. Snout to vent length (SVL) is used to assess tadpole growth as illustrated in Figure 2.

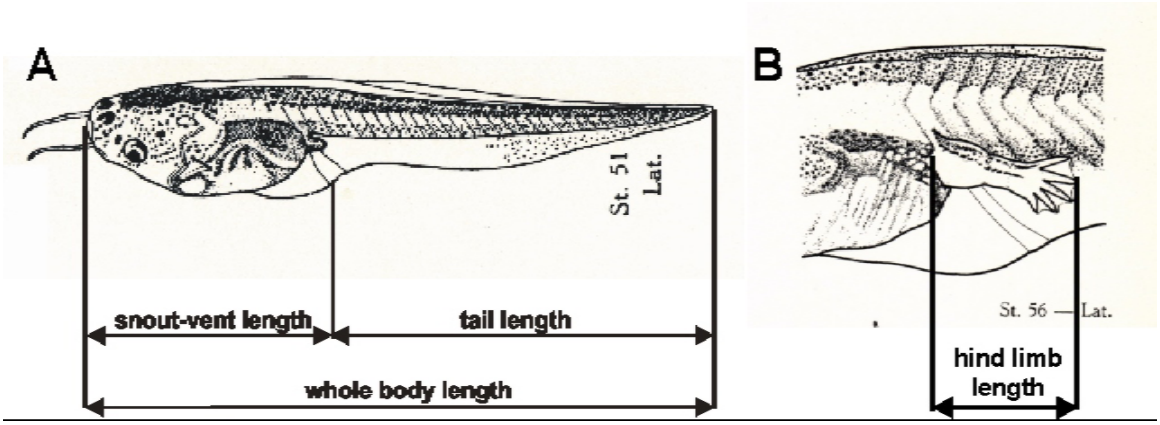


Figure 2. (A) Types of body length measurements and (B) Hind limb length measurements for X. laevis tadpoles (1).

Thyroid Gland Histology

39. While developmental stage and hind limb length are important endpoints to evaluate exposure-related changes in metamorphic development, developmental delay cannot, by itself, be considered a diagnostic indicator of anti-thyroidal activity. Some changes may only be observable by routine histopathological analysis. Diagnostic criteria include thyroid gland hypertrophy/atrophy, follicular cell hypertrophy, follicular cell hyperplasia, and as additional qualitative criteria: follicular lumen area, colloid quality and follicular cell height/shape. Severity grading (4 grades) should be reported. Information on obtaining and processing samples for histological analysis and for performing histologic analyses on tissue samples is available in "Amphibian Metamorphosis Assay: Part 1 - Technical guidance for morphologic sampling and histological preparation" and "Amphibian Metamorphosis Assay: Part 2 – Approach to reading studies, diagnostic criteria, severity grading and atlas" (9). Laboratories performing the assay for the first time(s) should seek advice from experienced pathologists for training purpose prior to undertaking histological analysis and evaluation of the thyroid gland. Overt and significant changes in apical endpoints indicating developmental acceleration or asynchrony may preclude the necessity to perform histopathological analysis of the thyroid glands. However, absence of overt morphological changes or evidence of developmental delay warrants histological analyses.

Mortality

40. All test tanks should be checked daily for dead tadpoles and the numbers recorded for each tank. The date, concentration and tank number for any observation of mortality should be recorded. Dead animals should be removed from the test tank as soon as observed. Mortality rates exceeding 10% may indicate inappropriate test conditions or toxic effects of the test chemical.

Additional Observations

41. Cases of abnormal behaviour and grossly visible malformations and lesions should be recorded. The date, concentration and tank number for any observation of abnormal behaviour, gross malformations or lesions should be recorded. Normal behaviour is characterised by the tadpoles being suspended in the water column with tail elevated above the head, regular rhythmic tail fin beating, periodic surfacing, operculating, and being responsive to stimulus. Abnormal behaviour would include, for example, floating on the surface, lying on the bottom of the tank, inverted or irregular swimming, lack of surfacing activity, and being nonresponsive to stimulus. In addition, gross differences in food consumption between treatments should be recorded. Gross malformations and lesions could include morphological abnormalities (e.g. limb deformities), hemorrhagic lesions, bacterial or fungal infections, to name a few. These determinations are qualitative and should be considered akin to clinical signs of disease/stress and made in comparison to control animals. If the occurrence or rate of occurrence is greater in exposed tanks than in the controls, then these should be considered as evidence for overt toxicity.

DATA AND REPORTING

Data Collection

42. All data should be collected using electronic or manual systems which conform to good laboratory practices (GLP). Study data should include:

Test chemical:

- Characterisation of the test chemical: physical-chemical properties; information on stability and biodegradability;
- Chemical information and data: method and frequency of preparation of dilutions. Test chemical information includes actual and nominal concentrations of the test chemical, and in some cases, non-parent chemical, as appropriate. Test chemical measurements may be required for stock solutions as well as for test solutions;
- Solvent (if other than water): justification of the choice of solvent, and characterisation of solvent (nature, concentration used);

Test conditions:

- Operational records: these consist of observations pertaining to the functioning of the test system and the supporting environment and infrastructure. Typical records include: ambient temperature, test temperature, photoperiod, status of critical components of the exposure system (e.g. pumps, cycle counters, pressures), flow

rates, water levels, stock bottle changes, and feeding records. General water quality parameters include: pH, DO, conductivity, total iodine, alkalinity, and hardness;
- Deviations from the test method: this information should include any information or narrative descriptions of deviations from the test method;

Results:
- Biological observations and data: these include daily observations of mortality, food consumption, abnormal swimming behaviour, lethargy, loss of equilibrium, malformations, lesions, etc. Observations and data collected at predetermined intervals include: developmental stage, hind limb length, snout vent length, and wet weight;
- Statistical analytical techniques and justification of techniques used; results of the statistical analysis preferably in tabular form;
- Histological data: these include narrative descriptions, as well as graded severity and incidence scores of specific observations, as detailed in the histopathology guidance document;
- Ad hoc observations: these observations should include narrative descriptions of the study that do not fit into the previously described categories.

Data reporting

43. Appendix 2 contains daily data collection spreadsheets that can be used as guidance for raw data entry and for calculations of summary statistics. Additionally, reporting tables are provided that are convenient for communicating summaries of endpoint data. Reporting tables for histological assessments can be found in Appendix 2.

Performance Criteria and Test Acceptability/Validity

44. Generally, gross deviations from the test method will result in unacceptable data for interpretation or reporting. Therefore, the following criteria in Table 4 have been developed as guidance for determining the quality of the test performed, the general performance of the control organisms.

Table 4. Performance criteria for the AMA.

Criterion	Acceptable limits
Test concentrations	Maintained at ≤ 20% CV(variability of measured test concentration) over the 21 day test
Mortality in controls	≤10% - mortality in any one replicate in the controls should not exceed 2 tadpoles
Minimum median developmental stage of controls at end of test	57
Spread of development stage in control group	The 10^{th} and the 90^{th} percentile of the development stage distribution should not differ by more than 4 stages
Dissolved Oxygen	≥ 40% air saturation*
pH	pH should be maintained between 6.5-8.5. The inter-replicate/inter-treatment differentials should

	not exceed 0.5.
Water temperature	22º ± 1ºC - the inter-replicate/inter-treatment differentials should not exceed 0.5 ºC
Test concentrations without overt toxicity	≥ 2
Replicate performance	≤ 2 replicates across the test can be compromised
Special conditions for use of a solvent	If a carrier solvent is used, both a solvent control and clean water control should be used and results reported
	Statistically significant differences between solvent control and water control groups are treated specially. See below for more information
Special conditions for static renewal system	Representative chemical analyses before and after renewal should be reported
	Ammonia levels should be measured immediately prior to renewal
	All water quality parameters listed in Table 1of Appendix 1 should be measured immediately prior to renewal
	Renewal period should not exceed 72 hours
	Appropriate feeding schedule (50% of the daily food ration of commercial tadpole feed)

*Aeration of water can be maintained through bubblers. It is recommended to set bubblers at levels that do not create undue stress on the tadpoles.

Test Validity

45. The following requirements should be met to deem a test acceptable/valid:

Valid experiment in a test determined to be negative for thyroid activity:

(1) For any given treatment (including controls), mortality cannot exceed 10%. For any given replicate, mortality cannot exceed three tadpoles, otherwise the replicate is considered compromised

(2) At least two treatment levels, with all four uncompromised replicates, should be available for analysis

(3) At least two treatment levels without overt toxicity should be available for analysis

Valid experiment in a test determined to be positive for thyroid activity:

(1) Mortality of no more than two tadpoles/replicate in the control group can occur

Decision logic for the conduct of the AMA

46. Decision logic was developed for the AMA to provide logical assistance in the conduct and interpretation of the results of the bioassay (see flow chart in Figure 3). The

decision logic, in essence, weighs the endpoints in that advanced development, asynchronous development and thyroid histopathology are weighed heavily, while delayed development, snout-vent length and wet body weight, parameters that can potentially be affected by general toxicity, are weighed less heavily.

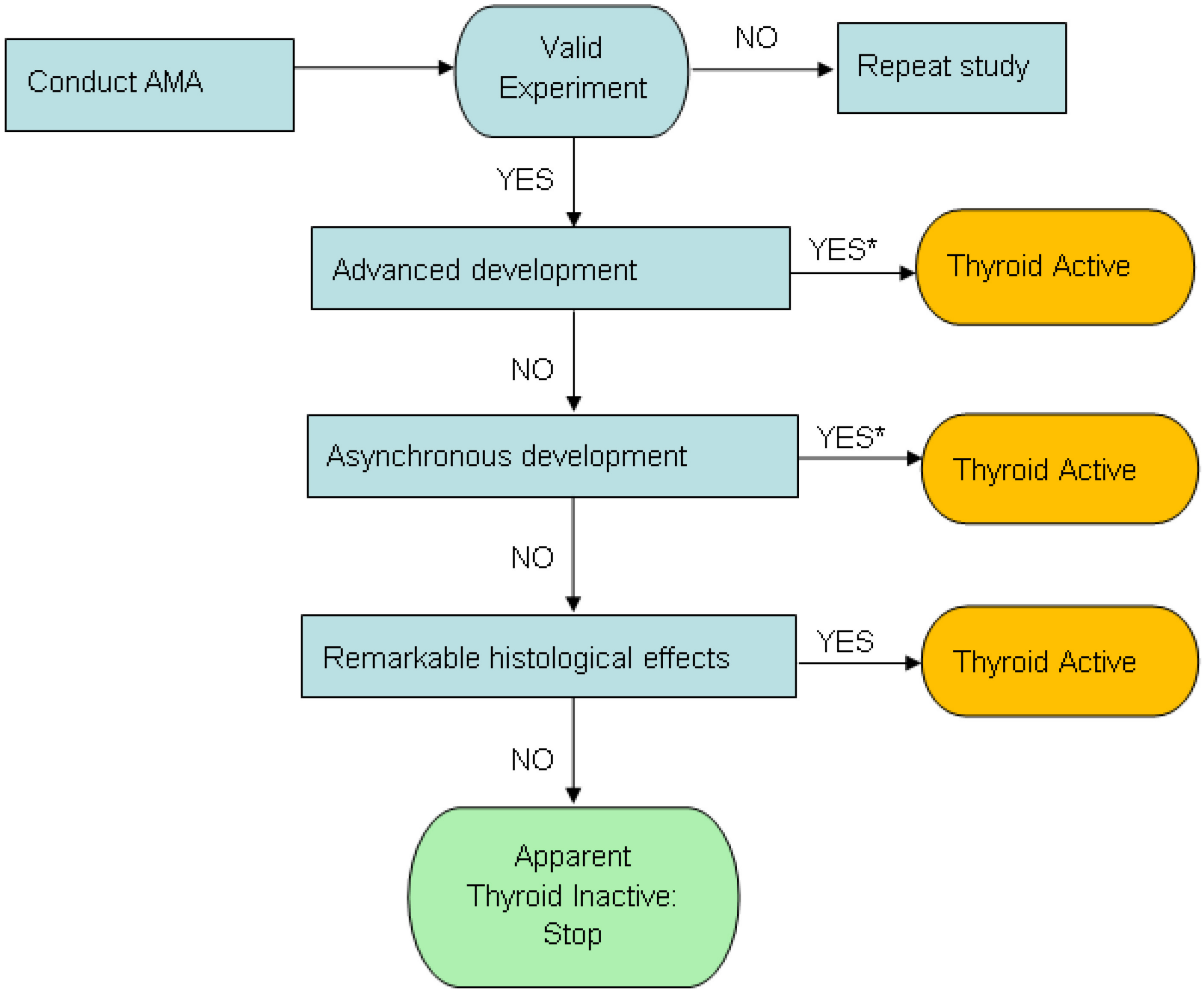


Figure 3. Decision logic for the conduct of the AMA.

*Histology may be required by some regulatory authorities despite significant differences in advanced and asynchronous development. The entity performing this test is encouraged to consult the necessary authorities prior to the performing the test to determine which endpoints are required.

Advanced development (determined using developmental stage, SVL and HLL)

47. Advanced development is only known to occur through effects which are thyroid hormone related. These may be peripheral tissue effects such as direct interaction with

the thyroid hormone receptor (such as with T4) or effects which alter circulating thyroid hormone levels. In either case, this is considered sufficient evidence to indicate that the chemical has thyroid activity. Advanced development is evaluated in one of two ways. First, the general developmental stage can be evaluated using the standardised approach detailed in Nieuwkoop and Faber (8). Second, specific morphological features may be quantified, such as hind limb length, at both days 7 and 21, which is positively associated with agonistic effects on the thyroid hormone receptor. If statistically significant advances in development or hind limb length occur, then the test indicates that the chemical is thyroid active.

48. The evaluation of test animals for the presence of accelerated development relative to the control population will be based on results of statistical analyses performed for the following four endpoints:

- hind limb length (normalised by SVL) on study day 7
- hind limb length (normalised by SVL) on study day 21
- developmental stage on study day 7
- developmental stage on study day 21.

49. Statistical analyses of hind limb length should be performed based on measurements of the length of the left hind limb. Hind limb length is normalised by taking the ratio hind limb length to snout-to-vent length of an individual. The mean of the normalised values for each treatment level are then compared. Acceleration of development is then indicated by a significant increase of mean hind limb length (normalised) in a chemical treatment group compared to the control group on study day 7 and/or study day 21 (see Appendix 3).

50. Statistical analyses of developmental stage should be performed based on determination of developmental stages according to the morphological criteria described by Nieuwkoop and Faber (8). Acceleration of development is indicated when the multi-quantal analysis detects a significant increase of developmental stage values in a chemical treatment group compared to the control group on study day 7 and/or study day 21.

51. In the AMA test method, a significant effect on any of the four endpoints mentioned above is regarded sufficient for a positive detection of accelerated development. That is, significant effects on hind limb length at a specific time point do not require corroboration by significant effects on hind limb length at the alternative time point nor by significant effects on developmental stage at this specific time point. In turn, significant effects on developmental stage at a specific time point do not require corroboration by significant effects at developmental stage on the alternative time point nor by significant effects on hind limb length at this specific time point. The weight of evidence for accelerated development will nevertheless increase if significant effects are detected for more than one endpoint.

Asynchronous development (determined using developmental stage criteria)

52. Asynchronous development is characterised by disruption of the relative timing of the morphogenesis or development of different tissues within a single tadpole. The

inability to clearly establish the developmental stage of an organism using the suite of morphological endpoints considered typical of any given stage indicates that the tissues are developing asynchronously through metamorphosis. Asynchronous development is an indicator of thyroid activity. The only known modes of action causing asynchronous development are through effects of chemicals on peripheral thyroid hormone action and/or thyroid hormone metabolism in developing tissues such as is observed with deiodinase inhibitors.

53. The evaluation of test animals for the presence of asynchronous development relative to the control population will be based on gross morphological assessment of test animals on study day 7 and study day 21.

54. The description of normal development of *Xenopus laevis* by Nieuwkoop and Faber (8) provides the framework for identifying a sequential order of normal tissue remodelling. The term "asynchronous development" refers specifically to those deviations in tadpole gross morphological development that disallow the definitive determination of a developmental stage according to the criteria of Nieuwkoop and Faber (8) because key morphological landmarks show characteristics of different stages.

55. As implicated by the term "asynchronous development", only cases showing deviations in the progress of remodelling of specific tissues relative to the progress of remodelling of other tissues should be considered. Some classical phenotypes include delay or absence of fore limb emergence despite normal or advanced development of hind limbs and tail tissues, or the precocious resorption of gills relative to the stage of hind limb morphogenesis and tail resorption. An animal will be recorded as showing asynchronous development if it cannot be assigned to a stage because it fails to meet a majority of the landmark developmental criteria for a given Niewkoop and Faber stage (8), or if there is extreme delay or acceleration of one or more key features (e.g. tail completely resorbed, but forelimbs not emerged). This assessment is performed qualitatively and should examine the full suite of landmark features listed by Nieuwkoop and Faber (8). However it is not necessary to record the developmental state of the various landmark features of animals being observed. Animals recorded as showing asynchronous development are not assigned to a Nieuwkoop and Faber (8) development stage.

56. Thus, a central criterion for designating cases of abnormal morphological development as "asynchronous development" is that the relative timing of tissue remodelling and tissue morphogenesis is disrupted whereas the morphology of affected tissues is not overtly abnormal. One example to illustrate this interpretation of gross morphological abnormalities is that retarded hind limb morphogenesis relative to development of other tissues will fulfil the criterion of "asynchronous development" whereas cases showing missing hind limbs, abnormal digits (e.g. ectrodactyly, polydactyly), or other overt limb malformations should not be considered as "asynchronous development".

57. In this context, the major morphological landmarks that should be evaluated for their coordinated metamorphic progress should include hind limb morphogenesis, fore limb

morphogenesis, fore limb emergence, the stage of tail resorption (particularly the resorption of the tail fin), and head morphology (e.g. gill size and stage of gill resorption, lower jaw morphology, protrusion of Meckel's cartilage).

58. Dependent on the mode of chemical action, different gross morphological phenotypes can occur. Some classical phenotypes include delay or absence of fore limb emergence in spite of normal or advanced development of hind limbs and tail tissues, precocious gill resorption relative to hind limb and tail remodelling.

Histopathology

59. If the chemical does not cause overt toxicity and does not accelerate development or cause asynchronous development, then histopathology of the thyroid glands is evaluated using the appropriate guidance document (9). Developmental retardation, in the absence of toxicity, is a strong indicator of anti-thyroid activity, but the developmental stage analysis is less sensitive and less diagnostic than the histopathological analysis of the thyroid gland. Therefore, conducting histopathological analyses of the thyroid glands is required in this case. Effects on thyroid gland histology have been demonstrated in the absence of developmental effects. If changes in thyroid histopathology occur, then the chemical is considered to be thyroid active. If no developmental delays or histological lesions are observed in the thyroid glands, then the chemical is considered to be thyroid inactive. The rationale for this decision is that the thyroid gland is under the influence of TSH and any chemical which alters circulating thyroid hormone sufficiently to alter TSH secretion will result in histopathological changes in the thyroid glands. Various modes and mechanisms of action can alter circulating thyroid hormone. So, while thyroid hormone level is indicative of a thyroid related effect, it is insufficient to determine which mode or mechanism of action is related to the response.

60. Because this endpoint is not amenable to basic statistical approaches, the determination of an effect associated with exposure to a chemical shall be made through expert opinion by a pathologist.

Delayed development (determined using developmental stage, HLL, BW, SVL)

61. Delayed development can occur through anti-thyroidal mechanisms and through indirect toxicity. Mild developmental delays coupled with overt signs of toxicity likely indicate a non-specific toxic effect. Evaluation of non-thyroidal toxicity is an essential element of the test to reduce the probability of false positive outcomes. Excessive mortality is an obvious indication that other toxic mechanisms are occurring. Similarly, mild reductions in growth, as determined by wet weight and/or SVL length, also suggest non-thyroidal toxicity. Apparent increases in growth are commonly observed with chemicals that negatively affect normal development. Consequently, the presence of larger animals does not necessarily indicate non-thyroidal toxicity. However, growth should never be solely relied upon to determine thyroid toxicity. Rather, growth, in conjunction with developmental stage and thyroid histopathology, should be used to determine thyroid activity. Other endpoints should also be considered in determining overt toxicity including oedema, haemorrhagic

lesions, lethargy, reduced food consumption, erratic/altered swimming behaviour, etc. If all test concentrations exhibit signs of overt toxicity, the test chemical should be re-evaluated at lower test concentrations before determining whether the chemical is potentially thyroid active or thyroid inactive.

62. Statistically significant developmental delays, in absence of other signs of overt toxicity, indicate that the chemical is thyroid active (antagonistic). In the absence of strong statistical responses, this outcome may be augmented with results from thyroid histopathology.

Statistical analyses

63. Statistical analyses of the data should preferably follow procedures described in the document Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application (11). For all continuous quantitative endpoints (HLL, SVL, wet weight) consistent with a monotone dose-response, the Jonckheere-Terpstra test should be applied in step-down manner to establish a significant treatment effect.

64. For continuous endpoints that are not consistent with a monotone dose-response, the data should be assessed for normality (preferably using the Shapiro-Wilk or Anderson-Darling test) and variance homogeneity (preferably using the Levene test). Both tests are performed on the residuals from an ANOVA. Expert judgment can be used in lieu of these formal tests for normality and variance homogeneity, though formal tests are preferred. Where non-normality or variance heterogeneity is found, a normalising, variance stabilising transformation should be sought. If the data (perhaps after a transformation) are normally distributed with homogeneous variance, a significant treatment effect is determined from Dunnett's test. If the data (perhaps after a transformation) are normally distributed with heterogeneous variance, a significant treatment effect is determined from the Tamhane-Dunnett or T3 test or from the Mann-Whitney-Wilcoxon U test. Where no normalising transformation can be found, a significant treatment effect is determined from the Mann-Whitney-Wilcoxon U test using a Bonferroni-Holm adjustment to the p-values. The Dunnett test is applied independently of any ANOVA F-test and the Mann-Whitney test is applied independently of any overall Kruskall-Wallis test.

65. Significant mortality is not expected but should be assessed from the step-down Cochran-Armitage test where the data are consistent with dose-response monotonicity, and otherwise from Fisher's Exact test with a Bonferroni-Holm adjustment.

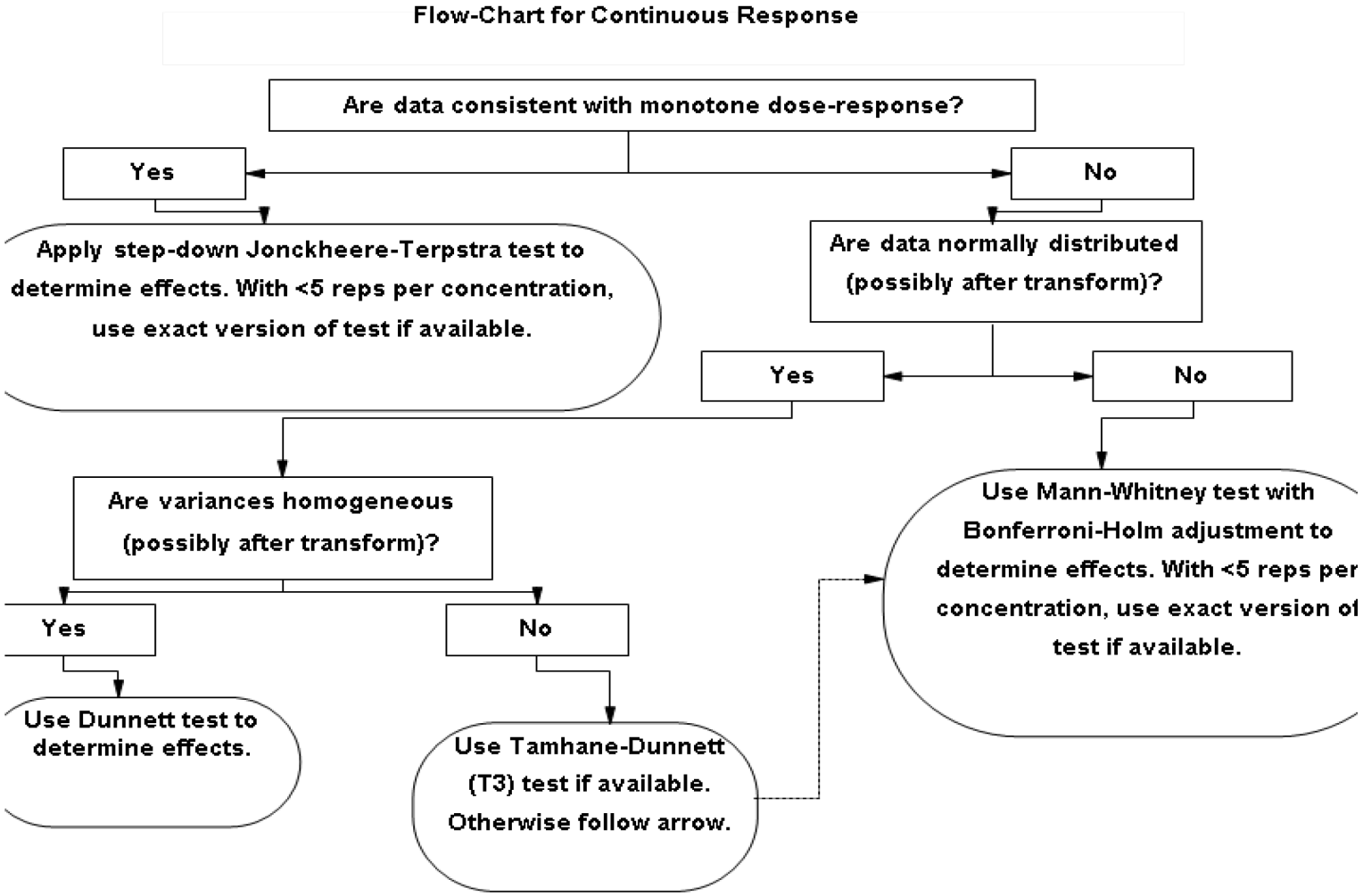
66. A significant treatment effect for developmental stage is determined from the step-down application of the Jonckheere-Terpstra test applied to the replicate medians. Alternatively, and preferably, the multi-quantal Jonckheere test from the 20^{th} to the 80^{th} percentile should be used for effect determination, as it takes into account changes to the distribution profile.

67. The appropriate unit of analysis is the replicate so the data consist of replicate medians if the Jonckheere-Terpstra or Mann-Whitney U test is used, or the replicate means if

Dunnett's test is used. Dose-response monotonicity can be assessed visually from the replicate and treatment means or medians or from formal tests such as previously described (11). With fewer than five replicates per treatment or control, the exact permutation versions of the Jonckheere-Terpstra and Mann-Whitney tests should be used if available. The statistical significance of all tests indicated is judged at the 0.05 significance level.

68. Figure 4 is a flow-chart for performing statistical tests on continuous data.

Figure 4. Flow-chart for statistical approaches for continuous response data.



Special data analysis considerations

Use of compromised treatment levels

69. Several factors are considered when determining whether a replicate or entire treatment demonstrates overt toxicity and should be removed from analysis. Overt toxicity is defined as >2 mortalities in any replicate that can only be explained by toxicity rather than technical error. Other signs of overt toxicity include haemorrhage, abnormal behaviours, abnormal swimming patterns, anorexia and any other clinical signs of disease. For sub-lethal signs of toxicity, qualitative evaluations may be necessary, and should always be made in reference to the clean water control group.

Solvent controls

70. The use of a solvent should only be considered as a last resort, when all other chemical delivery options have been considered. If a solvent is used, then a clean water control should be run in concert. At the termination of the test, an evaluation of the potential effects of the solvent should be performed. This is done through a statistical comparison of the solvent control group and the clean water control group. The most relevant endpoints for consideration in this analysis are developmental stage, SVL and wet weight, as these can be affected through non-thyroidal toxicities. If statistically significant differences are detected in these endpoints between the clean water control and solvent control groups, determine the study endpoints for the response measures using the clean water control. If there is no statistically significant difference between the clean water control and solvent control for all measured response variables, determine the study endpoints for the response measures using the pooled dilution-water and solvent controls.

Treatment groups achieving developmental stage 60 and above

71. After stage 60, tadpoles show a reduction in size and weight due to tissue resorption and reduction of absolute water content. Thus, measurements of wet weight and SVL cannot appropriately be used in statistical analyses for differences in growth rates. Therefore, wet weight and length data from organisms >NF60 should be censored and cannot be used in analyses of replicate means or replicate medians. Two different approaches could be used to analyse these growth-related parameters.

72. One approach is to consider only tadpoles with developmental stages lower or equal to stage 60 for the statistical analyses of wet weight and/or SVL. This approach is believed to provide sufficiently robust information about the severity of possible growth effects as long as only a small proportion of test animals are removed from the analyses (≤20%). If an increased number of tadpoles show development beyond stage 60 (≥20%) in one or more nominal concentration(s), then a two-factor ANOVA with a nested variance structure should be undertaken on all tadpoles to assess growth effects due to chemical treatments while taking into account the effect of late stage development on growth. Appendix 3 provides guidance on the two-factor ANOVA analysis of weight and length. .

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Appendix 1

Table 1: Experimental Conditions for the 21-day Amphibian Metamorphosis Assay

Test Animal		*Xenopus laevis* larvae	
Initial Larval Stage		Nieuwkoop and Faber stage 51	
Exposure Period		21 days	
Larvae Selection Criteria		Developmental stage and total length (optional)	
Test Concentrations		Minimum of 3 concentrations spanning approximately one order of magnitude	
Exposure Regime		Flow-through (preferred) and/or static-renewal	
Test System Flow-Rate		25 ml/min (complete volume replacement ca. every 2.7 h)	
Primary Endpoints / Determination Days		Mortality	Daily
		Developmental Stage	D 7 and 21
		Hind Limb Length	D 7 and 21
		Snout-Vent Length	D 7 and 21
		Wet Body Weight	D 7 and 21
		Thyroid Histology	D 21
Dilution Water / Laboratory Control		Dechlorinated tap water (charcoal-filtered) or the equivalent laboratory source	
Larval Density		20 larvae / test vessel (5 / l)	
Test Solution / Test Vessel		4-10 l (10-15 cm minimum water) / Glass or Stainless Steel test vessel (e.g., 22.5 cm x 14 cm x 16.5 cm)	
Replication		4 replicate test vessels / test concentration and control	
Acceptable Mortality Rate in Controls		≤ 10% per replicate test vessel	
Thyroid Fixation	Number Fixed	All tadpoles (5/replicate are evaluated initially)	

	Region	Head or whole body
	Fixation Fluid	Davidson's fixative
Feeding	Food	Sera Micron® or equivalent
	Amount / Frequency	See Table 1 for feeding regime using Sera Micron®
Lighting	Photoperiod	12 h Light : 12 h dark
	Intensity	600 to 2000 lux (Measured at Water Surface)
Water Temperature		22º ± 1ºC
pH		6.5 – 8.5
Dissolved Oxygen (DO) Concentration		>3.5 mg/l (>40% Air Saturation)
Analytical Chemistry Sample Schedule		Once / Week (4 Sample Events / Test)

Appendix 2

REPORTING TABLES FOR RAW DATA AND SUMMARY DATA

Table 1: General test chemical information

Chemical information

Enter test chemical, concentration units, and treatments

Test chemical:	
Concentration units:	
Treatment 1	
Treatment 2	
Treatment 3	
Tretament 4	

Date (day 0):		Enter date (mm/dd/yy)
Date (day 7):		Enter date (mm/dd/yy)
Date (day 21):		Enter date (mm/dd/yy)

Table 2: Raw data collection sheets for days 7 and 21

DAY X
DATE 00/00/00

ROW	Concentration TRT	Treatment Number TRT#	Replicate Number REP	Individual number IND	Individual Idendifier ID#	Developmental Stage STAGE	SVL Length (mm) BL	Hindlimb Length (mm) HLL	Whole Organism wet weight (mg) WEIGHT
1	0.00	1							
2	0.00	1							
3	0.00	1							
4	0.00	1							
5	0.00	1							
6	0.00	1							
7	0.00	1							
8	0.00	1							
9	0.00	1							
10	0.00	1							
11	0.00	1							
12	0.00	1							
13	0.00	1							
14	0.00	1							
15	0.00	1							
16	0.00	1							
17	0.00	1							
18	0.00	1							
19	0.00	1							
20	0.00	1							
21	0.00	2							
22	0.00	2							
23	0.00	2							
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25	0.00	2							
26	0.00	2							
27	0.00	2							
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71	0.00	4							
72	0.00	4							
73	0.00	4							
74	0.00	4							
75	0.00	4							
76	0.00	4							
77	0.00	4							
78	0.00	4							
79	0.00	4							
80	0.00	4							

Table 3: Calculated summaries for endpoint data from days 7 and 21

		Developmental Stage			**SVL (mm)**		**Hindlimb Length (mm)**		**Weight (mg)**	
TRT	REP	MIN	MEDIAN	MAX	MEAN	STD DEV	MEAN	STD DEV	MEAN	STD DEV
1	1	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
1	2	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
1	3	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
1	4	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
2	1	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
2	2	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
2	3	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
2	4	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
3	1	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
3	2	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
3	3	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
3	4	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
4	1	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
4	2	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
4	3	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
4	4	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Note: Cell calculations are associated with data entries into Table 2.

Table 4: Daily mortality data

Test Day	Date	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0	00/00/00																
1	#Value!																
2	#Value!																
3	#Value!																
4	#Value!																
5	#Value!																
6	#Value!																
7	#Value!																
8	#Value!																
9	#Value!																
10	#Value!																
11	#Value!																
12	#Value!																
13	#Value!																
14	#Value!																
15	#Value!																
16	#Value!																
17	#Value!																
18	#Value!																
19	#Value!																
20	#Value!																
21	#Value!																
Replicate count		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Treatment Count		0				0				0				0			

Note: Cell calculations are associated with data entries into Table 1.

Table 5: Water Quality Criteria

Exposure System (flow-through/static renewal):
Temperature:
Light intensity:
Light-dark cycle:
Food:
Feeding rate:
water pH:
Iodine concentration in test water:

Table 6: Summary chemistry data

Chemical Name:																					
Cas #:																					
Test Day	Date	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0	00/00/00																				
1	#Value!																				
2	#Value!																				
3	#Value!																				
4	#Value!																				
5	#Value!																				
6	#Value!																				
7	#Value!																				
8	#Value!																				
9	#Value!																				
10	#Value!																				
11	#Value!																				
12	#Value!																				
13	#Value!																				
14	#Value!																				
15	#Value!																				
16	#Value!																				
17	#Value!																				
18	#Value!																				
19	#Value!																				
20	#Value!																				
21	#Value!																				

Note: Cell calculations are associated with data entries into Table 1.

Table 7: Histopathology reporting tables for core criteria

Date: Chemical:

		Thyroid gland hypertrophy	Thyroid gland atrophy	Follicular cell hypertrophy	Follicular cell hyperplasia
Control Animal ID – replicate 1					
Control Animal ID – replicate 2					
Total:					

Pathologist:

		Thyroid gland hypertrophy	Thyroid gland atrophy	Follicular cell hypertrophy	Follicular cell hyperplasia
Dose Animal ID – replicate 1					
Dose Animal ID– replicate 2					
Total:					

		Thyroid gland hypertrophy	Thyroid gland atrophy	Follicular cell hypertrophy	Follicular cell hyperplasia
Dose Animal ID – replicate 1					
Dose Animal ID – replicate 2					
Total:					

		Thyroid gland hypertrophy	Thyroid gland atrophy	Follicular cell hypertrophy	Follicular cell hyperplasia
Dose Animal ID – replicate 1					
Dose Animal ID – replicate 2					
Total:					

Table 8: Additional histopathology criteria

Date: Chemical: Pathologist:

		Follicular lumen area increase	Follicular lumen area decrease
Control Animal ID – replicate 1			
Control Animal ID – replicate 2			
Total:			

		Follicular lumen area increase	Follicular lumen area decrease
Dose Animal ID -replicate 1			
Dose Animal ID -replicate 2			
Total:			

		Follicular lumen area increase	Follicular lumen area decrease
Dose Animal ID - replicate 1			
Dose Animal ID - replicate 2			
Total:			

		Follicular lumen area increase	Follicular lumen area decrease
Dose Animal ID -replicate 1			
Dose Animal ID - replicate 2			
Total:			

Table 9: Narrative descriptions for histopathological findings

Date:

Chemical:

Pathologist:

		Narrative description
Control Animal ID – replicate 1		
Control Animal ID – replicate 2		
Dose Animal ID - replicate 1		
Dose Animal ID - replicate 2		

Dose Animal ID – replicate 1		
Dose Animal ID - replicate 2		

Dose Animal ID – replicate 1		
Dose Animal ID – replicate 2		

Table 10: Summary reporting table template for day x (7 or 21) of the AMA

		Control				Dose 1					Dose 2					Dose 3				
Endpoint	**Replicate**	**Mean**	**SD**	**CV**	**N**	**Mean**	**SD**	**CV**	**N**	**p-value**	**Mean**	**SD**	**CV**	**N**	**p-value**	**Mean**	**SD**	**CV**	**N**	**p-value**
Hind Limb Length (mm)	1																			
	2																			
	3																			
	4																			
	Mean:																			
SVL (mm)	1																			
	2																			
	3																			
	4																			
	Mean:																			
Wet weight (mg)	1																			
	2																			
	3																			
	4																			
	Mean:																			

Table 11: Summary reporting table template for day x (7 or 21) developmental stage data for the AMA

		Control				Dose 1					Dose 2					Dose 3				
	Replicate	**Median**	**Min**	**Max**	**N**	**Median**	**Min**	**Max**	**N**	**p-value**	**Median**	**Min**	**Max**	**N**	**p-value**	**Median**	**Min**	**Max**	**Median**	**p-value**
Developmental Stage	1																			
	2																			
	3																			
	4																			
	Mean:																			

Appendix 3

ALTERNATIVE ANALYSIS OF WEIGHT AND LENGTH IN THE CASE OF LATE STAGE DEVELOPMENT EXCEEDING 20% OF TADPOLES IN ONE OR MORE CONCENTRATION(S)

If an increased number of tadpoles show development beyond stage 60 (≥20%) in one or more nominal concentration(s), then a two-factor ANOVA with a nested variance structure should be undertaken on all tadpoles to assess growth effects due to chemical treatments while taking into account the effect of late stage development on growth.

The proposal is to use all data but take into account the effect of late stage development. This can be done with a two-factor ANOVA with a nested variance structure. Define LateStage='Yes' for an animal if its developmental stage is 61 or greater. Otherwise, define LateStage='No'. Then a two-factor ANOVA with concentration and LateStage and their interaction can be done, with Rep(Conc) a random factor and Tadpole(Rep) another random effect. This still treats the rep as the unit of analysis and gives essentially the same results as a weighted analysis of rep*latestage means, weighted by the number of animals per mean. If the data violate the normality or variance homogeneity requirements of ANOVA, then a normalised rank-order transform can be done to remove that objection.

In addition to the standard ANOVA F-tests for the effects of Conc, LateStage, and their interactions, the interaction F-test can be "sliced" into two additional ANOVA F-test, one on the mean responses across concentrations for LateStage='No' and another on the mean responses across concentrations for LateStage='Yes.' Further comparisons of treatment means against control are done within each level of LateStage. A trend-type analysis can be done using appropriate contrasts or simple pairwise comparisons can be done if there is evidence of non-monotone dose-response within a level of the LateStage variable. A Bonferroni-Holm adjustment to the p-values is made only if the corresponding F-slice is not significant. This can be done in SAS and, presumably, other statistical software packages. Complications can arise when there are no late stage animals in some concentrations, but these situations can be handled in a straight-forward fashion.

Appendix 4

DEFINITIONS

Chemical: A substance or a mixture

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

C.39. Collembolan Reproduction Test in Soil

INTRODUCTION

1. This test method is equivalent to OECD test guideline (TG) 232 (2009). This test method is designed for assessing the effects of chemicals on the reproductive output of the collembolans in soil. It is based on existing procedures (1) (2). The parthenogenetic *Folsomia candida* and sexually reproducing *Folsomia fimetaria* are two of the most accessible species of Collembola, and they are culturable and commercially available. When specific habitats not covered by the two species need to be assessed the procedure is extensible also to other species of Collembola if they are able to fulfil the validity criteria of the test.

2. Soil-dwelling Collembola are ecologically relevant species for ecotoxicological testing. Collembolans are hexapods with a thin exoskeleton highly permeable to air and water, and represent arthropod species with a different route and a different rate of exposure compared to earthworms and enchytraeids.

3. Population densities of Collembola commonly reach 10^5 m^{-2} in soil and leaf litter layers in many terrestrial ecosystems (3) (4). Adults typically measure 0.5 - 5 mm, their contribution to total soil animal biomass and respiration is low, estimated between 1% and 5% (5). Their most important role may therefore be as potential regulators of processes through microbivory and microfauna predation. Springtails are prey animals for a wide variety of endogeic and epigeic invertebrates, such as mites, centipedes, spiders, Carabidae and rove beetles. Collembola contribute to decomposition processes in acidic soils where they may be the most important soil invertebrates besides enchytraeids, since earthworms and diplopods are typically absent.

4. *F. fimetaria* has a worldwide distribution and is common in several soil types ranging from sandy to loamy soils and from mull to mor soils. It is an eyeless, unpigmented collembolan. It has been recorded in agricultural soils all over Europe (6). It has an omnivorous feeding habit, including fungal hyphae, bacteria, protozoa and detritus in its food. It interacts through grazing with infections of plant pathogenic fungi (7) and may influence mycorrhiza, as is known to be the case for F. candida. As most collembolan species it reproduces sexually requiring the permanent presence of males for egg fertilisation.

5. *F. candida* is also distributed worldwide. Although it is not common in most natural soils, it often occurs in very high numbers in humus rich sites. It is an eyeless, unpigmented collembolan. It has a well-developed furca (jumping organ) and an active running movement and jumps readily if disturbed. The ecological role of *F. candida* is similar to the role of *F. fimetaria*, but the habitats are more organic rich soils. It reproduces parthenogenetically. Males may occur at less than 1 per thousand.

PRINCIPLE OF THE TEST

6. Synchronous adult (*F. fimetaria*) or juvenile (*F. candida*) Collembola are exposed to a range of concentrations of the test chemical mixed into a modified artificial soil (8) using a 5% organic matter content (or an alternative soil). The test scenario can be divided into two steps:

- A range-finding test, in case no sufficient information on toxicity is available, in which mortality and reproduction are the main endpoints assessed after 2 weeks for *F. fimetaria* and 3 weeks for *F. candida*.
- A definitive reproduction test in which the total number of juveniles produced by parent animals and the survival of parent animals are assessed. The duration of this definitive test is 3 weeks for *F. fimetaria* or 4 weeks for *F. candida*.

The toxic effect of the test chemical on adult mortality and reproductive output is expressed as LC_x and EC_x by fitting the data to an appropriate model by non-linear regression to estimate the concentration that would cause x % mortality or reduction in reproductive output, respectively, or alternatively as the NOEC/LOEC value (9).

INFORMATION ON THE TEST CHEMICAL

7. The physical properties, water solubility, the log K_{ow}, the soil water partition coefficient and the vapour pressure of the test chemical should preferably be known. Additional information on the fate of the test chemical in soil, such as the rates of photolysis and hydrolysis and biotic degradation, is desirable. Chemical identification of the test chemical according to IUPAC nomenclature, CAS-number, batch, lot, structural formula and purity should be documented when available.

8. This Test Method can be used for water soluble or insoluble chemicals. However, the mode of application of the test chemical will differ accordingly. The test method is not applicable to volatile chemicals, i.e. chemicals for which the Henry's constant or the air/water partition coefficient is greater than one, or chemicals for which the vapour pressure exceeds 0.0133 Pa at 25 °C.

VALIDITY OF THE TEST

9. The following criteria should be satisfied in the untreated controls for a test result to be considered valid:

- Mean adult mortality should not exceed 20% at the end of the test;
- The mean number of juveniles per vessel should be at least 100 at the end of the test;
- The coefficient of variation calculated for the number of juveniles should be less than 30% at the end of the definitive test.

REFERENCE CHEMICAL

10. A reference chemical should be tested at its EC_{50} concentration for the chosen test soil type either at regular intervals or possibly included in each test run to verify that the response of the test organisms in the test system are within the normal level. A suitable

reference chemical is boric acid, which should reduce reproduction by 50% (10) (11) at about 100 mg/kg dry weight soil for both species.

DESCRIPTION OF THE TEST

Test vessels and equipment

11. Containers capable of holding 30 g of moist soil are suitable test vessels. The material should either be glass or inert plastic (non-toxic). However, using plastic containers should be avoided if the test chemical exposure is decreased due to sorption. The test vessels should have a cross-sectional area allowing the actual soil depth within the test vessel to be 2-4 cm. The vessels should have lids (e.g. glass or polyethylene) that are designed to reduce water evaporation whilst allowing gas exchange between the soil and the atmosphere. The container should be at least partly transparent to allow light transmission.

12. Normal laboratory equipment is required, specifically the following:

- drying cabinet;
- stereo microscope;
- pH-meter and luxmeter;
- suitable accurate balances;
- adequate equipment for temperature control;
- adequate equipment for air humidity control (not essential if exposure vessels are covered by lids);
- temperature-controlled incubator or small room;
- forceps or a low-suction air flow device.

Preparation of the test soil

13. A modified artificial soil (8) is used with an organic matter content of 5%. Alternatively a natural soil could be used, as the artificial soil does not resemble natural soils. The recommended composition of the artificial soil is as follows (based on dry weights, dried to a constant weight at 105 °C):

- 5% sphagnum peat, air-dried and finely ground (a particle size of 2 ± 1 mm is acceptable);
- 20% kaolin clay (kaolinite content preferably above 30%);
- approximately 74% air-dried industrial sand (depending on the amount of $CaCO_3$ needed), predominantly fine sand with more than 50% of the particles between 50 and 200 microns. The exact amount of sand depends on the amount of $CaCO_3$ (see below), together they should add up to 75 %.
- 1.0% calcium carbonate ($CaCO_3$, pulverised, analytical grade) to obtain a pH of 6.0 ± 0.5; the amount of calcium carbonate to be added may depend principally on the quality/nature of the peat (see Note 1).

Note 1: The amount of $CaCO_3$ required will depend on the components of the soil substrate and should be determined by measuring the pH of pre-incubated moist soil sub-samples immediately before the test.

Note 2: It is recommended to measure the pH and optionally the C/N ratio, Cation Exchange Capacity (CEC) and organic matter content of the soil in order to enable a normalisation at a later stage and to better interpret the results.

Note 3: If required, e.g. for specific testing purposes, natural soils from unpolluted sites may also serve as test and/or culture substrate. However, if natural soil is used, it should be characterised at least by origin (collection site), pH, texture (particle size distribution), CEC and organic matter content and it should be free from any contamination. For natural soil it is advisable to demonstrate its suitability for a test and for achieving the test validity criteria before using the soil in a definitive test.

14. The dry constituents of the soil are mixed thoroughly (e.g. in a large-scale laboratory mixer). The maximum water holding capacity (WHC) of the artificial soil is determined in accordance with procedures described in Appendix 5. The moisture content of the testing soil should be optimised to attain a loose porous soil structure allowing collembolans to enter into the pores. This is usually between 40-60% of the maximum WHC.

15. The dry artificial soil is pre-moistened by adding enough de-ionised water to obtain approximately half of the final water content 2-7 days before the test start, in order to equilibrate/stabilise the acidity. For the determination of pH a mixture of soil and 1 M potassium chloride (KCl) or 0.01 M calcium chloride ($CaCl_2$) solution in a 1:5 ratio is used (according to Appendix 6). If the soil is more acidic than the required range, it can be adjusted by addition of an appropriate amount of $CaCO_3$. If the soil is too alkaline it can be adjusted by the addition of an inorganic acid harmless to collembolans.

16. The pre-moistened soil is divided into portions corresponding to the number of test concentrations (and reference chemical where appropriate) and controls used for the test. The test chemicals are added and the water content is regulated according to the paragraph 24.

Selection and preparation of test animals

17. The parthenogenetic *F. candida* is the recommended species, as in the ring testing of the test method (11) this species met the validity criteria for survival more often than *F. fimetaria*. If an alternative species is used, it should meet the validity criteria outlined in paragraph 9. At the start of the test the animals should be well fed and the age between 23-26 days for *F. fimetaria* and 9-12 days for *F. candida*. For each replicate, the number of *F. fimetaria* should be 10 males and 10 females, and for *F. candida* 10 females should be used (see Appendix 2 and Appendix 3). The synchronous animals are selected randomly from the dishes and their health and physical condition is checked for each batch added to a replicate. Each group of 10/20 individuals is added to a randomly selected test container and the big females of *F. fimetaria* are selected to ensure a proper distinction from the *F. fimetaria* males.

Preparation of test concentrations

18. Four methods of application of the test chemical can be used: 1) mixing the test

chemical into the soil with water as a carrier, 2) mixing the test chemical into the soil with an organic solvent as a carrier, 3) mixing the test chemical into the soil with sand as a carrier, or 4) application of the test chemical onto the soil surface. The selection of the appropriate method depends on the characteristic of the chemical and the purpose of the test. In general, mixing of the test chemical into the soil is recommended. However, application procedures that are consistent with the practical use of the test chemical may be required (e.g. spraying of liquid formulation or use of special pesticide formulations such as granules or seed dressings). The soil is treated before the collembolans are added, except when the test chemical is added to the soil surface collembolans should be allowed to enter the soil.

Test chemical soluble in water

19. A solution of the test chemical is prepared in deionised water in a quantity sufficient for all replicates of one test concentration. Each solution of test chemical is mixed thoroughly with one batch of pre-moistened soil before being introduced into the test vessel.

Test chemical insoluble in water

20. For chemicals insoluble in water, but soluble in organic solvents, the test chemical can be dissolved in the smallest possible volume of a suitable solvent (e.g. acetone) still ensuring proper mixing of the chemical in the soil and mixing it with a portion of the quartz sand required. Only volatile solvents should be used. When an organic solvent is used, all test concentrations and an additional solvent negative control should contain the same minimum amount of the solvent. Application containers should be left uncovered for a certain period to allow the solvent associated with the application of the test chemical to evaporate, ensuring no dissipation of the toxic chemical during this time.

Test chemical poorly soluble in water and organic solvents

21. For chemicals that are poorly soluble in water and organic solvents, quartz sand, which should be a part of the total sand added to the soil, is mixed with the quantity of test chemical to obtain the desired test concentration. This mixture of quartz sand and test chemical is added to the pre-moistened soil and thoroughly mixed after adding an appropriate amount of deionised water to obtain the required moisture content. The final mixture is divided between the test vessels. The procedure is repeated for each test concentration and an appropriate control is also prepared.

Application of the test chemical onto the soil surface

22. When the test chemical is a pesticide, it may be appropriate to apply it onto the soil surface by spraying. The soil is treated after the collembolans are added. The test containers are first filled with the moistened soil substrate, and the animals added and then the test containers are weighted. In order to avoid any direct exposure of the animals with the test chemical by direct contact, the test chemical is applied at least half an hour after introducing the Collembola. The test chemical should be applied to

the surface of the soil as evenly as possible using a suitable laboratory-scale spraying device to simulate spray application in the field. The application should take place at a temperature within ± 2 °C of variation and for aqueous solutions, emulsions or dispersions at a water application rate according to the risk assessment recommendations. The rate should be verified using an appropriate calibration technique. Special formulations like granules or seed dressings could be applied in a manner consistent with agricultural use. Food is added after spraying.

PROCEDURE

Test conditions

23. The test mean temperature should be 20 ± 1 °C with a temperature range of 20 ± 2 °C. The test is carried out under controlled light-dark cycles (preferably 12 hours light and 12 hours dark) with illumination of 400 to 800 lux in the area of the test vessels.

24. In order to check the soil humidity, the vessels are weighed at the beginning, in the middle and at the end of the test. Weight loss > 2% is replenished by the addition of de-ionised water. It should be noted that loss of water can be reduced by maintaining a high air-humidity (> 80%) in the test incubator.

25. The pH should be measured at the beginning and the end of both the range-finding test and the definitive test. Measurements should be made in one extra control sample and one extra sample of the treated (all concentrations) soil samples prepared and maintained in the same way as the test cultures, but without addition of the collembolans.

Test procedure and measurements

26. For each test concentration, an amount of test soil corresponding to 30 g fresh weight is placed into the test vessel. Water controls, without the test chemical, are also prepared. If a vehicle is used for application of the test chemical, one control series containing the vehicle alone should be run in addition to the test series. The solvent or dispersant concentration should be the same as that used in the test vessels containing the test chemical.

27. The individual springtails are carefully transferred into each test vessel (allocated randomly to the test vessels) and placed onto the surface of the soil. For efficient transfer of the animals, a low-suction air flow device can be used. The number of replicates for test concentrations and for controls depends on the test design used. The test vessels are positioned randomly in the test incubator and these positions are re-randomised weekly.

28. For the *F. fimetaria* test twenty adults, 10 males and 10 females, 23-26 days old should be used per test-vessel. On day 21 collembolans are extracted from the soil and counted. For *F. fimetaria* the gender are discriminated by size in the synchronised animal batch used for the test. Females are distinctively larger than the males (See Appendix 3)

29. For the *F. candida* test, ten 9-12 days old juveniles per test vessel should be used. On day 28, the collembolans are extracted from the soil and counted.

30. As a suitable food source, a sufficient amount, e.g. 2-10 mg, of granulated dried baker's yeast, commercially available for household use, is added to each container at the beginning of the test and after about 2 weeks.

31. At the end of the test, mortality and reproduction are assessed. After 3 weeks (*F. fimetaria*) or 4 weeks (*F. candida*), collembolans are extracted from the test soil (see Appendix 4) and counted (12). A collembolan is recorded as dead if not present in the extraction. The extraction and counting method should be validated. The validity includes extraction efficiency of juveniles greater than 95%, e.g. by adding a known number to soil.

32. Practical summary and timetable of the test procedure are described in Appendix 2.

Test design

Range-finding test

33. When necessary, a range-finding test is conducted with, for example, five test chemical concentrations of 0.1, 1.0, 10, 100, and 1000 mg/kg dry weight of soil and two replicates for each treatment and control. Additional information, from tests with similar chemicals or from literature, on mortality or reproduction of Collembola may also be useful in deciding on the range of concentrations to be used in the range-finding test.

34. The duration of the range-finding test is two weeks for *F. fimetaria* and 3 weeks for *F. candida* to ensure one clutch of juveniles has been produced. At the end of the test, mortality and reproduction of the Collembola are assessed. The number of adults and the occurrence of juveniles should be recorded.

Definitive test

35. For determination of the EC_x (e.g. EC_{10}, EC_{50}), twelve concentrations should be tested. At least two replicates for each test concentration treatment and six control replicates are recommended. The spacing factor may vary depending on the dose-response pattern.

36. For determination of the NOEC/LOEC, at least five concentrations in a geometric series should be tested. Four replicates for each test concentration treatment plus eight controls are recommended. The concentrations should be spaced by a factor not exceeding 1.8.

37. A combined approach allows for determination of both the NOEC/LOEC and ECx. For this combined approach, eight treatment concentrations in a geometric series should be used. Four replicates for each treatment plus eight controls are recommended. The concentrations should be spaced by a factor not exceeding 1.8.

38. If no effects are observed at the highest concentration in the range-finding test (i.e. 1000 mg/kg), the reproduction test can be performed as a limit test, using a test concentration of 1000 mg/kg and the control. A limit test will provide the opportunity to demonstrate that there is no statistically significant effect at the limit concentration. Eight replicates should be used for both the treated soil and the control.

DATA AND REPORTING

Treatment of results

39. The reproductive output is the main endpoint (e.g. the number of juveniles produced per test vessel). The statistical analysis, e.g. ANOVA procedures, compares treatments by Student t-test, Dunnett's test, or Williams' test. 95% confidence intervals are calculated for individual treatment means.

40. The number of surviving adults in the untreated controls is a major validity criterion and should be documented. As in the range-finding test, all other harmful signs should be reported in the final report as well.

LC_x and EC_x

41. EC_x-values, including their associated lower and upper 95% confidence limits for the parameter, are calculated using appropriate statistical methods (e.g. logistic or Weibull function, trimmed Spearman-Karber method, or simple interpolation). An EC_x is obtained by inserting a value corresponding to x% of the control mean into the equation found. To compute the EC_{50} or any other EC_x, the complete data set should be subjected to regression analysis. LC_{50} is usually estimated by probit analysis or similar analysis that takes into account the binomially distributed mortality data.

NOEC/LOEC

42. If a statistical analysis is intended to determine the NOEC/LOEC, per-vessel statistics (individual vessels are considered replicates) are necessary. Appropriate statistical methods should be used according to OECD Document 54 on the Current Approaches in the Statistical Analysis of Ecotoxicity Data: a Guidance to Application (9). In general, adverse effects of the test chemical compared to the control are investigated using one-tailed hypothesis testing at $p \leq 0.05$.

43. Normal distribution and variance homogeneity can be tested using an appropriate statistical test, e.g. the Shapiro-Wilk test and Levene test, respectively ($p \leq 0.05$). One-way Analysis of Variance (ANOVA) and subsequent multi-comparison tests can be performed. Multiple comparisons (e.g. Dunnett's test) or step-down trend tests (e.g. Williams' test) can be used to calculate whether there are significant differences ($p \leq 0.05$) between the controls and the various test chemical concentrations (selection of the recommended test according to OECD Document 54 (9)). Otherwise, non-parametric methods (e.g. Bonferroni-U-test according to Holm or Jonckheere-Terpstra trend test) could be used to determine the NOEC and the LOEC.

Limit test

44. If a limit test (comparison of control and one treatment only) has been performed and the prerequisites of parametric test procedures (normality, homogeneity) are fulfilled, metric responses can be evaluated by the Student test (t-test). The unequal-variance t-test (Welch t-test) or a non parametric test, such as the Mann-Whitney-U-test may be used, if these requirements are not fulfilled.

45. To determine significant differences between the controls (control and solvent control), the replicates of each control can be tested as described for the limit test. If these tests do not detect significant differences, all control and solvent control replicates may be pooled. Otherwise all treatments should be compared with the solvent control.

Test report

46. The test report should at least include the following information:

Test chemical
- the identity of the test chemical, batch, lot and CAS-number, purity;
- physico-chemical properties of the test chemical (e.g. log Kow, water solubility, vapour pressure, Henry's constant (H) and preferably information on the fate of the test chemical in soil) if available;
- the formulation of the test chemical and the additives should be specified if not the pure chemical is tested;

Test organisms
- identification of species and supplier of the test organisms, description of the breeding conditions and age range of test organisms;

Test conditions
- description of the experimental design and procedure;
- preparation details for the test soil; detailed specification if natural soil is used (origin, history, particle size distribution, pH, organic matter content);
- water holding capacity of the soil;
- description of the technique used to apply the test chemical to the soil;
- test conditions: light intensity, duration of light-dark cycles, temperature;
- a description of the feeding regime, the type and amount of food used in the test, feeding dates;
- pH and water content of the soil at the start and end of the test (control and each treatment);
- detailed description of the extraction method and extraction efficiency;

Test results
- the number of juveniles determined in each test vessel at the end of the test;
- number of adults and their mortality (%) in each test vessel at the end of the test;
- a description of obvious physiological or pathological symptoms or distinct changes in behaviour;
- the results obtained with the reference test chemical;
- the NOEC/LOEC values, LC_x for mortality and EC_x for reproduction (mostly LC_{50},

LC_{10}, EC_{50}, and EC_{10}) together with 95% confidence intervals. A graph of the fitted model used for calculation, its function equation and its parameters (See (9));
- all information and observations helpful for the interpretation of the results;
- power of the actual test if hypothesis testing is done (9);
- deviations from procedures described in this Test Method and any unusual occurrences during the test;
- validity of the test;
- for NOEC, when estimated, the minimal detectable difference.

LITERATURE

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Appendix 1

DEFINITIONS

The following definitions are applicable to this test method (in this test all effect concentrations are expressed as a mass of test chemical per dry mass of the test soil):

Chemical is a substance or a mixture.

NOEC (no observed effect concentration) is the test chemical concentration at which no effect is observed. In this test, the concentration corresponding to the NOEC, has no statistically significant effect ($p < 0.05$) within a given exposure period when compared with the control.

LOEC (lowest observed effect concentration) is the lowest test chemical concentration that has a statistically significant effect ($p < 0.05$) within a given exposure period when compared with the control.

ECx (Effect concentration for x% effect) is the concentration that causes an x% of an effect on test organisms within a given exposure period when compared with a control. For example, an EC_{50} is a concentration estimated to cause an effect on a test end point in 50% of an exposed population over a defined exposure period.

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

Appendix 2

MAIN ACTIONS AND TIMETABLE FOR PERFORMING A COLLEMBOLAN TEST

The steps of the test can be summarised as follows:

Time (day)	**Action**
-23 to -26	Preparation of synchronous *F. fimetaria* culture
-14	Prepare artificial soil (mixing of dry constituents) Check pH of artificial soil and adjust accordingly Measure max WHC of soil
-9 to -12	Preparation of synchronous *F. candida* culture
-2 to -7	Pre-moist soil
–1	Distribute juveniles into batches Prepare stock solutions and apply test chemical if solvent required
0	Prepare stock solutions and apply test chemical if solid chemical, water soluble or surface application is required. Measure soil pH and weigh the containers. Add food. Introduce collembolans.
14	Range-finding test *F. fimetaria*: Terminate test, extract animals, measure soil pH and loss of water (weight) Definitive tests: Measure moisture content and replenish water and add 2-10 mg yeast
21	Definitive *F. fimetaria* test: Terminate test, extract animals, measure soil pH and loss of water (weight) Range-finding *F. candida*: Terminate test, extract animals, measure soil pH and loss of water (weight)
28	Definitive *F. candida* test: Terminate test, extract animals, measure soil pH and loss of water (weight)

Appendix 3

GUIDANCE ON REARING AND SYNCHRONISATION OF *F. FIMETARIA* AND *F. CANDIDA*

The time and durations given in this guidance should be checked for each specific collembolan strain to ensure that timing will allow for sufficient synchronised juveniles. Basically, the incidence of oviposition after the adults are transferred to fresh substrate and egg hatching determines the appropriate day for egg collection and collection of synchronous juveniles.

It is recommended to have a permanent stock culture consisting of e.g. 50 containers/Petri dishes. The stock culture should be kept in a good feeding condition by weekly feeding, watering and removal of old food and carcasses. Too few collembolans on the substrate may result in inhibition by more fungal growth. If the stock culture is used for egg production too often, the culture may get fatigue. Signs of fatigue are dead adults and mould on the substrate. The remaining eggs from the production of synchronous animals can be used to rejuvenate the culture.

In a synchronous culture of *F. fimetaria*, males are distinguished from females primarily by size. Males are clearly smaller than females, and the walking speed of the males is faster than for females. Correct selection of the gender requires little practice and can be confirmed by microscopic inspection of the genital area (13).

1. Rearing

1.a. Preparation of culturing substrate

The culturing substrate is plaster of Paris (calcium sulphate) with activated charcoal. This provides a moist substrate, with the function of the charcoal being to absorb waste gases and excreta (14) (15). Different forms of charcoal may be used to facilitate observations of the Collembola. For example, powdered charcoal is used for *F. candida* and *F. fimetaria* (producing a black/grey plaster of Paris):

Substrate constituents:

- 20 ml of activated charcoal
- 200 ml of distilled water
- 200 ml of plaster of Paris

or

- 50 g of activated pulverized charcoal
- 260-300 ml of distilled water
- 400 g plaster of Paris.

The substrate mixture is allowed to set before use.

1.b. Breeding

Collembolans are held in containers such as Petri dishes (90 mm x 13 mm), with the bottom covered by a 0.5 cm layer of plaster /charcoal substrate. They are cultured at 20 ± 1 °C at a light-dark cycle of 12-12 hours (400-800 Lux). Containers are kept moist at all times ensuring that the relative humidity of the air within the containers is 100%. This can be guaranteed by presence of free water within the porous plaster, but avoiding generating a water film on the plaster surface. Water loss can be prevented by providing a humid ambient air. Any dead individuals should be removed from the containers, as should any mouldy food. To stimulate production of eggs it is necessary to transfer the adult animals to Petri dishes with newly prepared plaster of Paris/charcoal substrate.

1.c. Food source

Granulated dried baker's yeast is used as the sole food supply for both *F. candida* and *F fimetaria*. Fresh food is provided once or twice a week, to avoid moulding. It is placed directly on the plaster of Paris in a small heap. The mass of baker's yeast added should be adjusted to the size of the collembolan population, but as a general rule 2-15 mg is sufficient.

2. Synchronisation

The test should be performed with synchronised animals to obtain homogeneous test animals of the same instar and size. Furthermore, the synchronisation enables discrimination of *F. fimetaria* males and females from the age of 3 weeks and onwards based on sexual dimorphism, i.e. size differences. The procedure below is a suggestion on how to obtain synchronised animals (the practical steps are optional).

2.a. Synchronisation.

- Prepare containers with a 0.5 cm layer of plaster of Paris/charcoal substrate.
- For egg laying transfer 150-200 adult *F. fimetaria* and 50-100 *F. candida* from the best 15-20 containers of the stock culture with 4-8 weeks old substrate to the containers and feed them 15 mg baker's yeast. Avoid bringing juveniles together with adults as presence of juveniles may inhibit egg production.
- Keep the culture at 20 ± 1 °C (the mean should be 20 °C) and a light-dark cycle of 12-12 hours (400-800 Lux). Ensure that fresh food is available and the air is water saturated. Lack of food may lead the animals to defecate on the eggs resulting in fungal growth on the eggs or *F. candida* may cannibalise its own eggs. After 10 days the eggs are carefully collected with a needle and spatula and moved to “egg-paper” (small pieces of filter paper dipped in plaster of Paris/charcoal slurry) which is placed in a container with fresh plaster/charcoal substrate. A few grains of yeast are added to the substrate to attract the juveniles and make them leave the egg-paper. It is important that the egg-paper and substrate are humid, or the eggs will dehydrate. As an alternative, adult animals may be removed from the synchronisation culture boxes after producing eggs for 2 or 3 days.
- After three days most of the eggs on the egg-paper will have hatched, and some juveniles may be found under the egg-paper.
- To have evenly aged juveniles, the egg-paper with un-hatched eggs is removed from the Petri dish with forceps. The juveniles, now 0-3 days, stay in the dish and are fed baker's yeast. Un-hatched eggs are discharged.
- Eggs and hatched juveniles are cultured in the same manner as the adults. In particular for *F. fimetaria* the following measures should be taken: ensuring sufficient fresh

food, old moulding food is removed, after 1 week the juveniles are divided into new Petri dishes provided that the density is above 200.

2.b. Handling collembolans at test initiation

- 9-12 days old *F. candida* or the 23-26 days old *F. fimetaria* are collected, e.g. by suction, and released into a small container with moist plaster/charcoal substrate and their physical condition is checked under the binocular (injured and damaged animals are disposed). All steps should be done while keeping the collembolans in a moist atmosphere to avoid drought stress, e.g. by using wetted surfaces etc.
- Turn the container up-side down and knock on it to transfer the collembolans to the soil. Static electricity should be neutralised, otherwise the animals may just fly into the air, or stick to the side of the test container and dry out. An ioniser or a moist cloth below the container may be used for neutralisation.
- The food should be spread all over the soil surface and not just in one lump.
- During transportation and during the testing period it should be avoided to knock or otherwise physically disturb the test containers, as this may increase the compaction of the soil, and hamper the interaction between the collembolans.

3. Alternative Collembolan species

Other collembolan species may be selected for testing according to this test method such as Proisotoma minuta, Isotoma viridis, Isotoma anglicana, Orchesella cincta, Sinella curviseta, Paronychiurus kimi, Orthonychiurus folsomi, Mesaphorura macrochaeta. A number of prerequisites should be fulfilled in advance before using alternative species:

- They should be unequivocally identified;
- The rationale for the selection of the species should be given;
- It should be ensured that the reproductive biology is included in the testing phase so it will be a potential target during the exposure;
- The life-history should be known: age at maturation, duration of egg development, and instars subject to exposure;
- Optimal conditions for growth and reproduction should be provided by the test substrate and food supply;
- Variability should be sufficiently low for precise and accurate toxicity estimation.

Appendix 4

EXTRACTION AND COUNTING OF ANIMALS

1. Two methods of extraction can be performed.

1.a. First method: A controlled temperature gradient extractor based on principles by MacFadyen can be used (1). The heat coming from a heating element at the top of the extraction box (regulated through a thermistor placed on the surface of the soil sample). The temperature in the cooled liquid surrounding the collecting vessel is regulated through a thermistor situated at the surface of the collection box (placed below the soil core). The thermistors are connected to a programmable controlling unit which raises the temperature according to a pre-programmed schedule. Animals are collected in the cooled collecting box (2 °C) with a bottom layer of plaster of Paris/charcoal. Extraction is started at 25 °C and the temperature is increased automatically every 12 h by 5 °C and has a total duration of 48 hours. After 12 h at 40 °C the extraction is finished.

1.b. Second method: After the experimental incubation period the number of juvenile Collembola present is assessed by flotation. For that purpose the test is performed in the vessels of approximately 250 ml volume. At the end of the test approx. 200 ml of distilled water are added. The soil is gently agitated with a fine paintbrush to allow Collembola to float to the water surface. A small amount, approx. 0.5 ml, of black Kentmere photographic dye may be added to the water to aid counting by increasing the contrast between the water and the white Collembola. The dye is not toxic to Collembola.

2. Counting:

Counts of numbers may be carried out by eye or under a light microscope using a grid placed over the floatation vessel or by photographing the surface of each vessel and later counting the Collembola on enlarged prints or projected slides. Counts may also be performed using digital image processing techniques (12). All techniques should be validated.

Appendix 5

DETERMINATION OF THE MAXIMUM WHC OF THE SOIL

The following method for determining the maximum water holding capacity (WHC) of the soil has been found to be appropriate. It is described in Annex C of ISO DIS 11268-2 (Soil Quality - Effects of pollutants on earthworms (*Eisenia fetida*). Part 2: Determination of effects on reproduction).

Collect a defined quantity (e.g. 5 g) of the test soil substrate using a suitable sampling device (auger tube etc.). Cover the bottom of the tube with a wet piece of filter paper and then place it on a rack in a water bath. The tube should be gradually submerged until the water level is above to the top of the soil. It should then be left in the water for about three hours. Since not all water absorbed by the soil capillaries can be retained, the soil sample should be allowed to drain for a period of two hours by placing the tube onto a bed of very wet finely ground quartz sand contained within a covered vessel (to prevent drying). The sample should then be weighed, dried to constant mass at 105 °C. The water holding capacity (WHC) should be calculated as follows:

$$\text{WHC (in \% of dry mass)} = \frac{S - T - D}{D} \times 100$$

Where:

S = water-saturated substrate + mass of tube + mass of filter paper
T = tare (mass of tube + mass of filter paper)
D = dry mass of substrate

Appendix 6

DETERMINATION OF SOIL PH

The following method for determining the pH of a soil is based on the description given in ISO DIS 10390: Soil Quality – Determination of pH.

A defined quantity of soil is dried at room temperature for at least 12 h. A suspension of the soil (containing at least 5 grams of soil) is then made up in five times its volume of either a 1 M solution of analytical grade potassium chloride (KCl) or a 0.01 M solution of analytical grade calcium chloride ($CaCl_2$). The suspension is then shaken thoroughly for five minutes and then left to settle for at least 2 hours but not for longer than 24 hours. The pH of the liquid phase is then measured using a pH-meter that has been calibrated before each measurement using an appropriate series of buffer solutions (e.g. pH 4.0 and 7.0).

C.40. Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment

INTRODUCTION

1. This test method is equivalent to OECD Testing Guideline (TG) 233 (2010). It is designed to assess the effects of life-long exposure of chemicals on the freshwater dipteran *Chironomus* sp., fully covering the 1st generation (P generation) and the early part of the 2nd generation (F1 generation). It is an extension of the existing test methods C.28 (1) or C.27 (15) using a spiked-water exposure scenario or a spiked sediment scenario, respectively. It takes into account existing toxicity test protocols for *Chironomus riparius* and *Chironomus dilutus* (previously named *C. tentans* (2)) that have been developed in Europe and North America (3) (4) (5) (6) (7) (8) (9) and subsequently ring-tested (1) (7) (10) (11) (12). Other well documented chironomid species may also be used, e.g. *Chironomus yoshimatsui* (13) (14). The complete exposure duration is ca. 44 days for *C. riparius* and *C. yoshimatsui*, and –ca. 100 days for *C. dilutus*.

2. Both water and sediment exposure scenarios are described in this test method. The selection of an appropriate exposure scenario depends on the intended application of the test. The water exposure scenario, spiking of the water column, is intended to simulate a pesticide spray drift event and covers the initial peak concentration in surface waters. Water spiking is also useful for other types of exposure (including chemical spills), but not for accumulation processes within the sediment lasting longer than the test period. In that case, and also when run-off is the main entry route of pesticides into water bodies, a spiked sediment design may be more appropriate. If other exposure scenarios are of interest, the test design may be readily adapted. For example, if the distribution of the test chemical between the water phase and the sediment layer is not of interest and adsorption to the sediment has to be minimised, the use of surrogate artificial sediment (e.g. quartz sand) may be considered.

3. Chemicals that require testing of sediment-dwelling organisms may persist in sediment over long periods. Sediment-dwelling organisms may be exposed via a number of routes. The relative importance of each exposure route, and the time taken for each to contribute to the overall toxic effect, is dependent on the physical-chemical properties of the chemical. For strongly adsorbing chemicals or for chemicals covalently binding to sediment, ingestion of contaminated food may be a significant exposure route. In order not to underestimate the toxicity of highly lipophilic chemicals, the use of food added to the sediment before application of the test chemical may be considered (see paragraph 31). Therefore, it is possible to include all routes of exposure and all life stages.

4. Measured endpoints are the total number of adults emerged (for both 1st and 2nd generations), development rate (for both 1st and 2nd generations), sex ratio of fully emerged and alive adults (for both 1st and 2nd generations), number of egg ropes per female (1st generation only) and fertility of the egg ropes (1st generation only).

5. Formulated sediment is strongly recommended. Formulated sediment has several advantages over natural sediments:

- experimental variability is reduced because it forms a reproducible "standardised matrix" and the need to source uncontaminated clean sediment is eliminated;
- tests can be initiated at any time without encountering seasonal variability in the test sediment and there is no need to pre-treat the sediment to remove indigenous fauna;
- reduced cost compared to field collection of sufficient quantities required for routine testing;
- formulated sediment allows for comparisons of toxicity across studies and ranking chemicals accordingly (3).

6. Definitions used are given in Appendix 1.

PRINCIPLE OF THE TEST

7. First instar chironomid larvae are exposed to a concentration range of the test chemical in a sediment-water system. The test starts by placing first instar larvae (1^{st} generation) into test beakers containing spiked sediment or alternately the test chemical is spiked into the water after addition of the larvae. Chironomid emergence, time to emergence and sex ratio of the fully emerged and alive midges are assessed. Emerged adults are transferred to breeding cages, to facilitate swarming, mating and oviposition. The number of egg ropes produced and their fertility are assessed. From these egg ropes, first instar larvae of the 2^{nd} generation are obtained. These larvae are placed into freshly prepared test beakers (spiking procedure as for the 1^{st} generation) to determine the viability of the 2^{nd} generation through an assessment of their emergence, time to emergence and the sex ratio of the fully emerged and alive midges (a schematic presentation of the life-cycle test is provided in Appendix 5). All data are analysed either by a regression model to estimate the concentration that would cause X% reduction in the relevant endpoint, or by using hypothesis testing to determine a No Observed Effect Concentration (NOEC). The latter requires a comparison of treatment responses with the appropriate control responses using statistical tests. It should be noted that in the spiked water scenario, in case of fast degrading chemicals, the later life stages of each generation (e.g. pupal phase) might be exposed to a considerably lower concentration level in the overlying water than the 1^{st} instar larvae. If this is a concern, and a comparable exposure level for each life stage is needed, the following amendments of the test method might be considered:

- parallel runs with spiking at different life stages, or
- repeated spiking (or overlying water renewal) of the test system during both test phases (1st and 2nd generation), whereby the spiking (renewal) intervals should be adjusted to the fate characteristics of the test chemical.

Such amendments are only feasible in the spiked water scenario, but not in the sediment spiked scenario.

INFORMATION ON THE TEST CHEMICAL

8. The water solubility of the test chemical, its vapour pressure and log K_{ow}, measured or calculated partitioning into sediment and stability in water and sediment should be

known. A reliable analytical method for the quantification of the test chemical in overlying water, pore water and sediment with known and reported accuracy and limit of detection should be available. Useful information includes the structural formula and purity of the test chemical. Chemical fate of the test chemical (e.g. dissipation, abiotic and biotic degradation, etc.) is also useful. Further guidance for testing chemicals with physical-chemical properties that make them difficult to perform the test is provided in (16).

REFERENCE CHEMICALS

9. Reference chemicals may be tested periodically as a means of assuring that the sensitivity of the laboratory population has not changed. As with daphnids it would be sufficient to perform a 48-h acute test (following 17). However, until a validated acute guideline is available a chronic test according to Chapter C.28 of this Annex may be considered. Examples of reference toxicants used successfully in ring-tests and validation studies are: lindane, trifluralin, pentachlorophenol, cadmium chloride and potassium chloride. (1) (3) (6) (7) (18).

VALIDITY OF THE TEST

10. For the test to be valid the following conditions apply:

- the mean emergence in the control treatment should be at least 70% at the end of the exposure period for both generations (1) (7);
- for *C. riparius* and *C. yoshimatsui*, 85% of the total emerged adult midges from the control treatment in both generations should occur between 12 and 23 days after the insertion of the first instar larvae into the vessels; for *C. dilutus*, a period of 20 to 65 days is acceptable;
- the mean sex ratio of fully emerged and alive adults (as female or male fraction) in the control treatment of both generations should be at least 0.4, but not exceed 0.6;
- for each breeding cage the number of egg ropes in the controls of the 1st generation should be at least 0.6 per female added to the breeding cage;
- the fraction of fertile egg ropes in each breeding cage of the controls of the 1st generation should be at least 0.6;
- at the end of the exposure period for both generations, pH and the dissolved oxygen concentration should be measured in each vessel. The oxygen concentration should be at least 60% of the air saturation value (ASV[1]), and the pH of overlying water should be between 6 and 9 in all test vessels;
- the water temperature should not differ by more than ± 1.0°C.

DESCRIPTION OF THE METHOD

[1] At 20°C under standard atmospheric pressure the ASV in freshwater equals 9.1 mg/l (60% equals 5.46 mg/l)

Test vessels and breeding cages

11. The larvae are exposed in 600 ml glass beakers measuring ca. 8.5 cm in diameter (see Appendix 5). Other vessels are suitable, but they should guarantee a suitable depth of overlying water and sediment. The sediment surface should be sufficient to provide 2 to 3 cm^2 per larvae. The ratio of the depth of the sediment layer to the depth of the overlying water should be ca. 1:4. Breeding cages (minimum 30 cm in all three dimensions) with a gauze (mesh size ca. 1 mm) on the top and one side of the cage as a minimum should be used (see Appendix 5). In each cage a 2 l crystallising dish, containing test water and sediment, is placed for oviposition. Also for the crystallising dish, the ratio of the depth of the sediment layer to the depth of the overlying water should be around 1:4. After egg ropes are collected from the crystallising dish they are placed into a 12-well microtiter plate (one rope per well containing at least 2.5 ml water from the spiked crystallising dish) after which the plates are covered with a lid to prevent significant evaporation. Other vessels suitable for keeping the egg ropes may also be used. With the exception of the microtiter plates, all test vessels and other apparatus that will come into contact with the test system should be made entirely of glass or other chemically inert material (e.g. Polytetrafluoroethylene).

Selection of species

12. The species to be used in the test is preferably *Chironomus riparius*. *C. yoshimatsui* may also be used. *C. dilutus* is also suitable but more difficult to handle and requires a longer test period. Details of culturing methods are given in Appendix 2 for *C. riparius*. Information on culture conditions are also available for *C. dilutus* (5) and *C. yoshimatsui* (14). Identification of the species should be confirmed before testing but is not required prior to every test if the organisms come from an in-house culture.

Sediment

13. Formulated sediment (also called reconstituted, artificial or synthetic sediment) should preferably be used. However, if natural sediment is used, it should be characterised (at least pH, organic carbon content, determination of other parameters such as C/N ratio and granulometry are also recommended) and should be free from any contamination and other organisms that may compete with, or consume chironomid larvae. It is also recommended, before testing, that sediments are conditioned for seven days under test conditions. The following formulated sediment, as described in (1), is recommended (1) (20) (21):

    a. 4-5% (dry weight) peat: as close to pH 5.5 to 6.0 as possible; it is important to use peat in powder form, finely ground (particle size ≤ 1 mm) and only air dried;

    b. 20% (dry weight) kaolin clay (kaolinite content preferably above 30%);

    c. 75-76% (dry weight) quartz sand (fine sand should predominate with more than 50 per cent of the particles between 50 and 200 μm);

    d. Deionised water is added to obtain moisture of the final mixture in the range of 30–50%;

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

f. Organic carbon content of the final mixture should be 2% (± 0.5%) and is to be adjusted by the use of appropriate amounts of peat and sand, according to (a) and (c).

14. The source of peat, kaolin clay and sand should be known. The sediment components should be checked for the absence of chemical contamination (e.g. heavy metals, organochlorine compounds, organophosphorous compounds). An example for the preparation of the formulated sediment is described in Appendix 3. Mixing of dry constituents is also acceptable if it is demonstrated that after addition of overlying water a separation of sediment constituents (e.g. floating of peat particles) does not occur, and that the peat or the sediment is sufficiently conditioned.

Water

15. Any water which conforms to the chemical characteristics of acceptable dilution water as listed in Appendices 2 and 4 is suitable as test water. Any suitable water, natural water (surface or ground water), reconstituted water (see Appendix 2) or dechlorinated tap water are acceptable as culturing water and test water, if chironomids will survive in it for the duration of the culturing and testing without showing signs of stress. At the start of the test, the pH of the test water should be between 6 and 9 and the total hardness not higher than 400 mg/l as $CaCO_3$. However, if there is an interaction suspected between hardness ions and the test chemical, lower hardness water should be used (and thus, Elendt Medium M4 should not be used in this situation). The same type of water should be used throughout the entire study. The water quality characteristics listed in Appendix 4 should be measured at least twice a year or when it is suspected that these characteristics may have changed significantly.

Stock solutions - Spiked water

16. a. Test concentrations are calculated on the basis of water column concentrations, i.e. the water overlying the sediment. Test solutions of the chosen concentrations are usually prepared by dilution of a stock solution. Stock solutions should preferably be prepared by dissolving the test chemical in test water. The use of solvents or dispersants may be required in some cases in order to produce a suitably concentrated stock solution. Examples of suitable solvents are acetone, ethylene glycol monoethyl ether, ethylene glycol dimethylether, dimethylformamide and triethylene glycol. Dispersants which may be used are Cremophor RH40, Tween 80, methylcellulose 0.01% and HCO-40. The solubilising agent concentration in the final test medium should be minimal (i.e. ≤ 0.1 ml/l) and should be the same in all treatments. When a solubilising agent is used, it should have no significant effects on survival as revealed by a solvent control in comparison with a negative (water) control. However, every effort should be made to avoid the use of such materials.

Stock solutions - Spiked sediment

16. b. Spiked sediments of the chosen concentration are usually prepared by addition of a solution of the test chemical directly to the 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 10 g of fine quartz sand for each test vessel. The solvent is allowed to evaporate and it should be totally removed from sand; the sand is then mixed with the suitable amount of sediment. Only agents which volatilise readily can be used to solubilise, disperse or emulsify the test chemical. It should be born in mind that the sand provided by the test chemical and sand mixture, should be taken into account when preparing 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. If necessary, subsamples can be analysed to determine degree of homogeneity.

TEST DESIGN

17. The test design relates to the selection of the number and spacing of the test concentrations, the number of vessels at each concentration, the number of larvae per vessel, the number of crystallising dishes and breeding cages. Designs for $EC_{x,}$ NOEC and a limit test are described below.

Design for analysis by regression

18. The effect concentration ($EC_{x,}$) and the concentration range over which the effect of the test chemical is of interest, should be spanned by the test, such that the endpoint is not extrapolated outside the bounds of the data generated. Extrapolation much below the lowest or above the highest concentration should be avoided. A preliminary range-finding test according to Test Methods C.27 or C.28 may be helpful for selecting a suitable range of test concentrations.

19. For an EC_x approach, at least five concentrations and eight replicates for each concentration are required. For each concentration two breeding cages should be used (A and B). The eight replicates are divided into two groups of four replicates to serve each breeding cage. This merger of replicates is necessary due to the number of midges needed in the cage for sound reproduction assessments. However, the 2nd generation has eight replicates again, which are initiated from the exposed populations in the breeding cages. The factor between concentrations should not be greater than two (an exception could be made in cases when the dose response curve has a shallow slope). The number of replicates at each treatment can be reduced to six (three for each breeding case) if the number of test concentrations with different responses is increased. Increasing the number of replicates or reducing the size of the test concentration intervals tends to lead to narrower confidence intervals around the EC_X.

Design for estimation of a NOEC

20. For a NOEC approach, five test concentrations with at least eight replicates (4 for each breeding cage, A and B) should be used and the factor between concentrations should not be greater than two. The number of replicates should be sufficient to ensure adequate statistical power to detect a 20% difference from the control at the 5% level of significance ($\alpha = 0.05$). For the development rate, fecundity and fertility an analysis

of variance (ANOVA) is usually appropriate, followed by Dunnett's test or Williams' test (22-25). For the emergence ratio and sex ratio the Cochran-Armitage, Fisher's exact (with Bonferroni correction), or Mantel-Haentzal tests may be appropriate.

Limit test

21. A limit test may be performed (one test concentration and control(s)) if no effects are observed in the optional preliminary range-finding test up to a maximum concentration. The purpose of the limit test is to indicate that any toxic effects of the test chemical are found at levels greater than the limit concentration tested. For water, 100 mg/l and for sediment 1000 mg/kg (dry weight) are suggested. Usually, at least eight replicates for both the treatment and control are necessary. Adequate statistical power to detect a 20% difference from the control at the 5% level of significance (α = 0.05) should be demonstrated. With metric responses (e.g. development rate), the t-test is a suitable statistical method if data meet the requirements of this test (normality, homogeneous variances). An unequal-variance t-test or a non-parametric test, such as the Wilcoxon-Mann-Whitney test may be used, if these requirements are not fulfilled. With the emergence ratio, Fisher's exact test is appropriate.

PROCEDURE

Conditions of exposure

Preparation of the water-sediment system (water spiking)

22. a. Formulated sediment (see paragraphs 13-14 and Appendix 3) is added to each test vessel and crystallising dish to form a layer of at least 1.5 cm (for the crystallising dish it may be somewhat lower) but maximally 3 cm. Water (see paragraph 15) is added so that the ratio of the depth of the sediment layer and the depth of the water does not exceed 1:4. After preparation of the test vessels the sediment-water system should be left under gentle aeration for approximately seven days prior to addition of the first instar larvae of the 1st or 2nd generation (see paragraph 14 and Appendix 3). The sediment-water system of the crystallising dishes is not aerated during the test, since they do not need to support larval survival (before hatching the egg ropes are already collected). To avoid separation of sediment ingredients and re-suspension of fine material during addition of test water in the water column, the sediment can be covered with a plastic disc while water is poured onto it. The disc is removed immediately afterwards. Other devices may also be appropriate.

Preparation of the water-sediment system (spiked sediment)

22. b. The spiked sediments prepared according to paragraph 16b are placed in the vessels and crystallising dish and overlying water is added to produce a sediment-water volume ratio of 1:4. The depth of the sediment layer should be in the range of 1.5 to 3 cm (it may be somewhat lower for the crystallising dish). To avoid separation of sediment ingredients and re-suspension of fine material during addition of test water in the water column, the sediment can be covered with a plastic disc while water is poured onto it, and the disc removed immediately afterwards. Other devices may also be appropriate. Once the spiked sediment with overlying water has been prepared, it is

desirable to allow partitioning of the test chemical from the sediment to the aqueous phase (4) (5) (7) (18). This should preferably be done under the conditions of temperature and aeration used in the test. Appropriate equilibration time is sediment and chemical specific, and can be in the order of hours to days and in rare cases up to five weeks. As this would leave time for degradation of many chemicals, equilibrium is not awaited but an equilibration period of 48 hours is recommended. However, when the degradation half-life of the chemical in sediment is known to be long (see paragraph 8), the equilibration time may be extended. At the end of this further equilibration period, the concentration of the test chemical should be measured in the overlying water, the pore water and the sediment, at least at the highest concentration and a lower one (see paragraph 38). These analytical determinations of the test chemical allow for calculation of a mass balance and expression of results based on measured concentrations.

23. Test vessels should be covered (e.g. by glass plates). If necessary, during the study the water levels may be topped up to the original volume in order to compensate for evaporation. This should be performed using distilled or deionised water to prevent any build-up of salts. Crystallising dishes in the breeding cages are not covered and may, but do not need to be adjusted to compensate for water loss during the test period, since the egg ropes are only in contact with the water for about one day and the dishes are only used during a short phase of the test.

Addition of test organisms

24. Four to five days before adding the first instar larvae for the 1st generation, egg masses should be taken from the culture and placed in small vessels in culture medium. Aged medium from the stock culture or freshly prepared medium may be used. In any case, a small amount of food, e.g. a few droplets of filtrate from a finely ground suspension of flaked fish food, should be added to the culture medium (see Appendix 2). Only freshly laid egg masses should be used. Normally, the larvae begin to hatch a couple of days after the eggs are laid (2 to 3 days for *C. riparius* at 20°C and 1 to 4 days for *C. dilutus* at 23°C and *C. yoshimatsui* at 25°C) and larval growth occurs in four instars, each of 4-8 days duration. First instar larvae (maximum 48 h post hatching) should be used in the test. The instar stage of larvae can potentially be checked using head capsule width (7).

25. Twenty first instar larvae for the 1st generation are allocated randomly to each test vessel containing the sediment-water system, using a blunt pipette. Aeration of the water is stopped whilst adding larvae to test vessels and should remain so for 24 hours following addition of larvae (see paragraph 32). According to the test design used (see paragraphs 19 and 20), the number of larvae used per concentration is at least 120 (6 replicates per concentration) for the EC_X approach and 160 for the NOEC approach (8 replicates per concentration). In the spiked sediment design, exposure starts with the addition of the larvae.

Spiking the overlying water

26. Twenty-four hours after adding the first instar larvae for the 1st generation, the test chemical is spiked into the overlying water column, and slight aeration is again

supplied (for possible amendments of the test design, see paragraph 7). Small volumes of the test chemical stock solutions are applied below the surface of the water using a pipette. The overlying water should then be mixed with care not to disturb the sediment. In the spiked water design, exposure starts with the spiking of the water (i.e. one day after addition of the larvae).

Collecting emerged adults

27. Emerged midges of the 1st generation are collected at least once, but preferably twice a day (see point 36) from the test vessels using an aspirator, exhauster or similar device (see Appendix 5). Special care should be taken not to damage the adults. The collected midges from four test vessels within one treatment are released into a breeding cage to which they had been previously assigned. At the day of first (male) emergence, crystallising dishes are spiked by pipetting a small volume of the test chemical stock solution below the water surface (spiked water design). The overlying water should then be mixed with care not to disturb the sediment. The concentration of test chemical in the crystallising dish is nominally the same as in the treatment vessels which are assigned to that specific breeding cage. For the spiked sediment design, the crystallising dishes are prepared at around day 11 after the start of the exposure (i.e. addition of the 1st generation larvae) so that they can equilibrate for about 48 hours before the first egg ropes are produced.

28. Egg ropes are collected from the crystallising dish in the breeding cage using tweezers or a blunt pipette. Each egg rope is placed into a vessel containing culture medium from the crystallising dish it was collected from (e.g. a well of a 12-well micro-plate together with at least 2.5 ml of medium). The vessels with the egg ropes are covered with a lid to prevent significant evaporation. Egg ropes are kept for observation for at least six days after they have been produced so that they can be classified as fertile or infertile.
    For starting the 2nd generation, at least three but preferably six fertile egg ropes are selected from each breeding cage and together with some food allowed to hatch. These egg ropes should have been produced at the peak of oviposition, which normally occurs around test day 19 in the controls. Ideally, the 2nd generation of all treatments is initiated on the same day, but due to chemical related effects on larval development, this may not always be possible. In such a case, the higher concentrations may be initiated later than the lower treatments and the (solvent) control.

29. a. In the spiked water design, the sediment-water system for the 2nd generation is prepared by spiking the test chemical into the overlying water column ca. 1 hour before adding the first instar larvae to the test vessels. Small volumes of the test chemical solutions are applied below the surface of the water using a pipette. The overlying water should then be mixed with care not to disturb the sediment. After spiking, slight aeration is supplied.

29. b. In the spiked sediment design, the exposure vessels containing the sediment-water system for the 2nd generation are prepared in the same way as for the 1st generation.

30. Twenty first instar larvae (maximum 48 h post hatching) of the 2nd generation are

allocated randomly to each test vessel containing the spiked sediment-water system, using a blunt pipette. Aeration of the water should be stopped while adding the first instar larvae to the test vessels and remain so for another 24 hours after addition of the larvae. According to the test design used (see paragraphs 19 and 20), the number of larvae used per concentration is at least 120 (6 replicates per concentration) for the EC_X approach and 160 for the NOEC approach (8 replicates per concentration).

Food

31. It is necessary to feed the larvae in the test vessels, preferably daily or at least three times per week. Fish-food (a suspension in water or finely ground food, e.g. Tetra-Min or Tetra-Phyll; see details in Appendix 2) of 0.25 - 0.5 mg (0.35 - 0.5 mg for C. yoshimatsui) per larvae per day is an adequate amount of food for young larvae during the first 10 days of their development. Slightly more food may be necessary for older larvae: 0.5 – 1.0 mg per larvae per day should be sufficient for the rest of the test. The food ration should be reduced in all treatments and control if fungal growth is seen or if mortality is observed in controls. If fungal development cannot be stopped the test should be repeated.

The toxicological relevance of exposure via ingestion is generally higher in chemicals with a high affinity for organic carbon or chemicals covalently binding to the sediment. Hence, when testing chemicals with such properties, the amount of food necessary to ensure survival and natural growth of the larvae may be added to the formulated sediment before the stabilisation period, depending on the regulatory demand. To prevent deterioration of the water quality, plant material should be used instead of fish food, e.g. addition of 0.5% (dry weight) finely ground leaves of stinging nettle (*Urtica dioica*), mulberry (*Morus alba*), white clover (*Trifolium repens*), spinach (*Spinacia oleracea*) or other plant material (*Cerophyl* or *α*-cellulose). Addition of the complete ration of an organic food source to the sediment before spiking is not trivial with respect to water quality and biological performance (21), nor a standardised method, but recent studies provide indications that this method works (19) (26). Adult midges in the breeding cage need no feeding normally, but fecundity and fertility are enhanced when a cotton wool pad soaked in a saturated sucrose solution is offered as a food source for emerged adults (34).

Incubation conditions

32. Gentle aeration of the overlying water in the test vessels is supplied 24 hours after addition of the first instar larvae of both generations and is continued throughout the test (care should be taken that the dissolved oxygen concentration does not fall below 60% of ASV). Aeration is provided through a glass Pasteur pipette of which the outlet is fixed 2-3 cm above the sediment layer giving a few bubbles/sec. When testing volatile chemicals, consideration should be given not to aerate the sediment-water system, while at the same time the validity criterion of minimal 60% ASV (paragraph 10) should be fulfilled. Further guidance is provided in (16).

33. The test with *C. riparius* is conducted at a constant temperature of 20°C (± 2°C). For *C. dilutus* and *C. yoshimatsui*, recommended temperatures are 23°C and 25°C (± 2°C), respectively. A 16 hours photoperiod is used and the light intensity should be 500 to

1000 lux. For the breeding cages an additional one hour dawn and dusk phase may be included.

Exposure duration

34. Spiked water design: The exposure period of the 1st generation starts when the test chemical is spiked into the overlying water of the test vessels (which is one day after insertion of the larvae – for possible amendments of the exposure design, see paragraph 7). Exposure of the 2nd larval generation starts immediately, since they are inserted into a sediment-water system that has been already spiked. The maximum exposure duration for the 1st generation is 27 days and for the 2nd generation 28 days (the 1st generation larvae spend one day in the vessels without exposure) for *C. riparius* and *C. yoshimatsui*. Considering the overlap, the complete test duration is approximately 44 days. For *C. dilutus*, maximum exposure durations are 64 and 65 days, for the 1st and 2nd generation, respectively. The total duration is approximately 100 days.

Spiked sediment design: exposure starts with the addition of the larvae and is maximum 28 days for both generations for *C. riparius* and *C. yoshimatsui* and maximum 65 days for both generations for *C. dilutus*.

Observations

Emergence

35. Development time and the total number of fully emerged and alive male and female midges are determined for both generations. Males are easily identified by their plumose antennae and thin body posture.

36. Test vessels of both generations should be observed at least three times per week to make visual assessment of any abnormal behaviour of the larvae (e.g. leaving sediment, unusual swimming), compared to the control. During the period of emergence, which starts about 12 days after insertion of the larvae for *C. riparius* and *C. yoshimatsui* (after 20 days for *C. dilutus*), emerged midges are counted and sexed at least once, but preferably twice a day (early morning and late afternoon). After identification, the midges of the 1st generation are carefully removed from the vessels and transferred to a breeding cage. Midges of the 2nd generation are removed and killed after identification. Any egg ropes deposited in the test vessels of the 1st generation should be collected individually and transferred with at least 2.5 ml native water to 12-well microplates (or other suitable vessels) which are covered with a lid to prevent significant evaporation. The number of dead larvae and visible pupae that have failed to emerge should also be recorded. Examples of a breeding cage, test vessel and exhauster are provided in Appendix 5.

Reproduction

37. Effects on reproduction are assessed via the number of egg ropes produced by the 1st generation of midges and the fertility of these egg ropes. Once per day the egg ropes are collected from the crystallising dish that is placed in each breeding container. The egg ropes should be collected and transferred with at least 2.5 ml native water to a 12-

wells microplate (one egg rope in each well) or other suitable vessels, which are covered with a lid to prevent significant evaporation. The following characteristics are documented for each egg rope: day of production, size (normal, i.e. 1.0 ± 0.3 cm or small; typically ≤ 0.5 cm), and structure (normal = banana-form with spiralled egg string or abnormal, e.g. unspiralled egg string) and fertility (fertile or infertile). Over the course of six days after it was produced the fertility of an egg rope is assessed. An egg rope is considered fertile when at least one third of the eggs hatch. The total number of females added to the breeding cage is used to calculate the number of egg ropes per female and the number of fertile egg ropes per female. If required, the number of eggs in an egg rope can be estimated non-destructively by using the ring count method (detailed in 32 and 33).

Analytical measurements

Concentration of the test chemical

38. As a minimum, samples of the overlying water, pore water and the sediment should be analysed at the start of exposure (in case of water spiking preferably one hour after application) and at the end of the test, at the highest concentration and a lower one. This applies to vessels from both generations. From the crystallising dishes in the breeding cage only the overlying water is analysed, since this is what the egg ropes come into contact with (for the spiked sediment design an analytical confirmation of the sediment concentration may be considered). Further measurements of sediment, pore water or overlying water during the test may be conducted if deemed necessary. These determinations of test chemical concentration inform on the behaviour/partitioning of the test chemical in the water-sediment system. Sampling of sediment and pore water at the start and during the test (see paragraph 39) requires additional test vessels to perform analytical determinations. Measurements in sediment in the spiked water design 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, type of application, organic carbon content of sediment), or if measured concentrations in the overlying water are shown to remain within 80 to 120% of the nominal or measured initial concentrations..

39. When intermediate measurements are made (e.g. at day 7 and/or 14) and if the analysis needs large samples which cannot be taken from test vessels without influencing the test system, analytical determinations should be performed on samples from additional test vessels treated in the same way (including the presence of test organisms) but not used for biological observations.

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

Physical-chemical parameters

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

DATA AND REPORTING

Treatment of results

42. The purpose of this life-cycle test is to determine the effect of the test chemical on the reproduction and, for two generations, the development rate and the total number of fully emerged and alive male and female midges. For the emergence ratio data of males and females should be pooled. If there are no statistically significant differences between the sensitivities in the development rate of the separate sexes, male and female results may be pooled for statistical analysis.

43. Effect concentrations expressed as concentrations in the overlaying water (for spiked water) or in the sediment (for spiked sediment), are usually calculated based on measured concentrations at the beginning of the exposure (see paragraph 38). Therefore, for spiked water, the concentrations typically measured at the beginning of the exposure in the overlying water of the vessels for both generations and those of the crystallising dishes are averaged for each treatment. For spiked sediment, the concentrations typically measured at the beginning of the exposure in the vessels for both generations (and optionally those of the crystallising dishes) are averaged for each treatment.

44. To compute a point estimate, i.e. an EC_x, the per-vessel and per-breeding cage statistics may be used as true replicates. In calculating a confidence interval for any EC_x the variability among vessels should be taken into account, or it should be shown that this variability is so small that it can be ignored. When the model is fitted by Least Squares, a transformation should be applied to the per-vessel statistics in order to improve the homogeneity of variance. However, EC_x values should be calculated after the response is transformed back to the original value (31).

45. When the statistical analysis aims at determining the NOEC by hypothesis testing, the variability among vessels needs to be taken into account, which is guaranteed by using ANOVA methods (e.g. Williams' and Dunnett's test procedures). Williams' test would be appropriate when a monotonic dose-response is expected in theory and Dunnett's test would be appropriate where the monotonicity hypothesis does not hold. Alternatively, more robust tests (27) can be appropriate in situations where there are violations of the usual ANOVA assumptions (31).

Emergence ratio

46. Emergence ratios are quantal data, and can be analysed by the Cochran-Armitage test applied in a step-down manner where a monotonic dose-response is expected and these data are consistent with this expectation. If not, a Fisher's exact or Mantel-Haentzal test with Bonferroni-Holm adjusted p-values can be used. If there is evidence of

greater variability between replicates within the same concentration than a binomial distribution would indicate (often referenced to as "extra-binomial" variation), then a robust Cochran-Armitage or Fisher exact test such as proposed in (27), should be used.

The sum of live midges (males plus females) emerged per vessel, n_e, is determined and divided by the number of larvae introduced, n_a:

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

where:

ER	=	emergence ratio
n_e	=	number of live midges emerged per vessel
n_a	=	number of larvae introduced per vessel (normally 20)

When n_e is larger than n_a (i.e. when unintentionally more than the foreseen number of larvae where introduced) n_a should be made equal to n_e.

47. An alternative approach that is most appropriate for large sample sizes, when there is extra binomial variance, is to treat the emergence ratio as a continuous response and use procedures consistent with these *ER* data. A large sample size is defined here as the number emerged and the number not emerging both exceeding five, on a per replicate (vessel) basis.

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

49. For emergence ratios, EC_x-values are calculated using regression analysis (e.g. probit, logit or Weibull models (28)). If regression analysis fails (e.g. when there are less than two partial responses), other non-parametric methods such as moving average or simple interpolation can be used.

Development rate

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

analysis (e.g. (29) (30)). A NOEC for the mean development rate can be determined via ANOVA methods, e.g. Williams or Dunnett's test. Since males emerge earlier than females, i.e. have a higher development rate, it makes sense to calculate the development rate for each gender separately in addition to that for the total midges.

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

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

where:

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

$$x_i = {}^{1}\Big/_{day_i - \frac{l_i}{2}}$$

where:

day_i	:	inspection day (days since introduction of the larvae)
l_i	:	length of inspection interval i (days, usually 1 day)

Sex ratio

52. Sex ratios are quantal data and should therefore be evaluated by means of a Fisher's exact test or other appropriate methods. The natural sex ratio of *C. riparius* is one, i.e. males and females are equally abundant. For both generations the sex ratio data should be treated identically. Since the maximum number of midges per vessel (i.e. 20) is too low for a meaningful statistical analysis, the total number of fully emerged and alive midges for each gender is summed over all vessels of one treatment. These untransformed data are tested against the (solvent) control or pooled control data in a 2 x 2 contingency table.

Reproduction

53. Reproduction, as fecundity, is calculated as the number of egg ropes per female. More specific, the total number of egg ropes produced in a breeding cage is divided by the total number of alive and undamaged females added to that cage. A NOEC for fecundity can be determined via ANOVA methods, e.g. Williams or Dunnett's test.

54. Fertility of the egg ropes is used to quantify the number of fertile egg ropes per female.

The total number of fertile egg ropes produced in a breeding cage is divided by the total number of alive and undamaged females added to that cage. A NOEC for fertility can be determined via ANOVA methods, e.g. Williams or Dunnett's test.

Test report

55. The test report should provide the following information:

Test chemical:

- physical nature and physical-chemical properties (water solubility, vapour pressure, log K_{ow}, partition coefficient in soil (or in sediment if available), stability in water and sediment etc.);
- chemical identification data (common name, chemical name, structural formula, CAS number, etc.) including purity and analytical method for the quantification of the test chemical.

Test species:

- test organisms used: species, scientific name, source of organisms and breeding conditions;
- information on how the egg masses and larvae were handled;
- information on handling of the emerged adults of the 1st generation with the help of an exhauster etc (see Appendix 5)
- age of the test organisms at the time of insertion into the test vessels of the 1st and 2nd generation.

Test conditions:

- sediment used, i.e. natural or formulated (artificial) sediment;
- natural sediment: location and description of sediment sampling site, including, if possible, contamination history; sediment characteristics: pH, organic carbon content, C/N ratio and granulometry (if appropriate).
- formulated sediment: preparation, ingredients and characteristics (organic carbon content, pH, moisture, etc. measured at the start of the test);
- preparation of the test water (if reconstituted water is used) and characteristics (oxygen concentration, pH, hardness, etc. measured at the start of the test);
- depth of sediment and overlaying water for the test vessels and crystallising dishes;
- volume of overlying and pore water; weight of wet sediment with and without pore water for the test vessels and the crystallising dishes;
- test vessels (material and size);
- crystallising dishes (material and size);
- breeding cages (material and size)
- method of preparation of stock solutions and test concentrations for the test vessels and crystallising dishes;
- application of the test chemical into the test vessels and crystallising dishes: test concentrations, number of replicates and solvents if needed;
- incubation conditions for the test vessels: temperature, light cycle and intensity, aeration (bubbles per second);
- incubation conditions for the breeding cages and the crystallising dishes: temperature, light cycle and intensity;
- incubation conditions for the egg ropes in the micro plates (or other vessels): temperature, light cycle and intensity:

- detailed information on feeding including type of food, preparation, amount and feeding regime.

Results:

- nominal test concentrations, measured test concentrations and the results of all analyses to determine the concentration of the test chemical in the test vessels and crystallising dishes;
- water quality within the test vessels and crystallising dishes, i.e. pH, temperature, dissolved oxygen, hardness and ammonia;
- replacement of evaporated test water for the test vessels, if any;
- number of emerged male and female midges per vessel and per day for the 1^{st} and 2^{nd} generation;
- sex ratio of fully emerged and alive midges per treatment for the 1^{st} and 2^{nd} generation
- number of larvae which failed to emerge as midges per vessel for the 1^{st} and 2^{nd} generation;
- percentage/fraction of emergence per replicate and test concentration (male and female midges pooled) for the 1^{st} and 2^{nd} generation;
- mean development rate of fully emerged and alive midges per replicate and treatment rate (male and female midges separate and also pooled) for the 1^{st} and 2^{nd} generation;
- number of egg ropes deposited in the crystallising dishes per breeding cage and day;
- characteristics of each egg rope (size, shape and fertility);
- fecundity – total number of egg ropes per total number of females added to the breeding cage;
- fertility – total number of fertile egg ropes per total number of females added to the breeding cage;
- estimates of toxic endpoints e.g. EC_x (and associated confidence intervals), NOEC and the statistical methods used for its determination;
- discussion of the results, including any influence on the outcome of the test resulting from deviations from this test method.

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Appendix 1

DEFINITIONS

For the purpose of this test method the following definitions are used:

Chemical is a substance or a mixture.

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

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

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

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

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

Appendix 2

RECOMMENDATIONS FOR CULTURE OF *CHIRONOMUS RIPARIUS*

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

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

Dilution water

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

Feeding larvae

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

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

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

Feeding emerged adults

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

Emergence

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

Egg masses

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

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

Set-up of new culture vessels

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

Preparation of test solutions "M4" and "M7"

12. Elendt (1990) has described the "M4" medium. The "M7" medium is prepared as the "M4" medium except for the substances indicated in Table 1, for which concentrations are four times lower in "M7" than in "M4". The test solution should not be prepared according to Elendt and Bias (1990) for the concentrations of $NaSiO_3 \cdot 5H_2O$, $NaNO_3$, KH_2PO_4 and K_2HPO_4 given for the preparation of the stock solutions are not adequate.

Preparation of the "M7"-medium

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

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

Stock solutions (I)	Amount (mg) made up to 1 litre of deionised water	To prepare the combined stock solution (II): mix the following amounts (ml) of stock solutions (I) and make up to 1 litre of deionised water		Final concentrations in test solutions (mg/l)	
		M4	M7	M4	M7
H_3BO_3 (1)	57190	1.0	0.25	2.86	0.715
$MnCl_2 \cdot 4H_2O$ (1)	7210	1.0	0.25	0.361	0.090
LiCl (1)	6120	1.0	0.25	0.306	0.077
RbCl (1)	1420	1.0	0.25	0.071	0.018
$SrCl_2 \cdot 6H_2O$ (1)	3040	1.0	0.25	0.152	0.038
NaBr (1)	320	1.0	0.25	0.016	0.004
$Na_2MoO_4 \cdot 2H_2O$ (1)	1260	1.0	0.25	0.063	0.016
$CuCl_2 \cdot 2H_2O$ (1)	335	1.0	0.25	0.017	0.004
$ZnCl_2$	260	1.0	1.0	0.013	0.013
$CaCl_2 \cdot 6H_2O$	200	1.0	1.0	0.010	0.010
KI	65	1.0	1.0	0.0033	0.0033
Na_2SeO_3	43.8	1.0	1.0	0.0022	0.0022
NH_4VO_3	11.5	1.0	1.0	0.00058	0.00058
$Na_2EDTA \cdot 2H_2O$ (1)(2)	5000	20.0	5.0	2.5	0.625
$FeSO_4 \cdot 7H_2O$ (1)(2)	1991	20.0	5.0	1.0	0.249

(1) These substances differ in M4 and M7, as indicated above.
(2) These solutions are prepared individually, then poured together and autoclaved immediately.

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

	Amount made up to 1 litre of deionised water (mg)	Amount of macro nutrient stock solutions added to prepare medium M4 and M7 (ml/l)	Final concentrations in test solutions M4 and M7 (mg/l)
$CaCl_2 \cdot 2H_2O$	293800	1.0	293.8

$MgSO_4 \cdot 7H_2O$	246600	0.5	123.3
KCl	58000	0.1	5.8
$NaHCO_3$	64800	1.0	64.8
$NaSiO_3 \cdot 9H_2O$	50000	0.2	10.0
$NaNO_3$	2740	0.1	0.274
KH_2PO_4	1430	0.1	0.143
K_2HPO_4	1840	0.1	0.184

Table 3: Vitamin stock solution for medium M4 and M7

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

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

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Appendix 3

PREPARATION OF FORMULATED SEDIMENT

Sediment composition

The composition of the formulated sediment should be as follows:

Constituent	Characteristics	% of sediment dry weight
Peat	Sphagnum moss peat, as close to pH 5.5-6.0 as possible, no visible plant remains, finely ground (particle size ≤ 1 mm) and air dried	4 - 5
Quartz sand	Grain size: > 50% of the particles should be in the range of 50-200 μm	75 - 76
Kaolinite clay	Kaolinite content ≥ 30%	20
Organic carbon	Adjusted by addition of peat and sand	2 (± 0.5)
Calcium carbonate	$CaCO_3$, pulverised, chemically pure	0.05 - 0.1
Water	Conductivity ≤ 10 μS/cm	30 - 50

Preparation

The peat is air dried and ground to a fine powder. A suspension of the required amount of peat powder in deionised water is prepared using a high-performance homogenising device. The pH of this suspension is adjusted to 5.5 ± 0.5 with $CaCO_3$. The suspension is conditioned for at least two days with gentle stirring at 20 ± 2°C, to stabilise pH and establish a stable microbial component. pH is measured again and should be 6.0 ± 0.5. Then the peat suspension is mixed with the other constituents (sand and kaolin clay) and deionised water to obtain an homogeneous sediment with a water content in a range of 30–50 per cent of dry weight of the sediment. The pH of the final mixture is measured once again and is adjusted to 6.5 to 7.5 with $CaCO_3$ if necessary. Samples of the sediment are taken to determine the dry weight and the organic carbon content. Then, before it is used in the chironomid toxicity test, it is recommended that the formulated sediment be conditioned for seven days under the same conditions which prevail in the subsequent test.

Storage

The dry constituents for preparation of the artificial sediment may be stored in a dry

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

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Appendix 4

CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

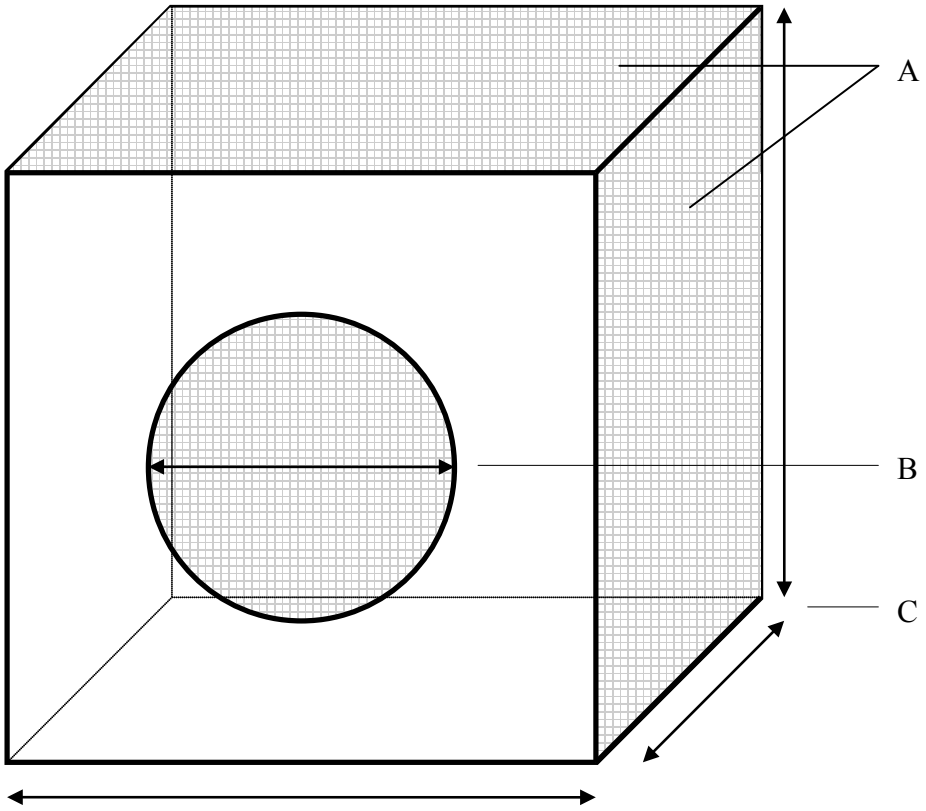
CONSTITUENT	CONCENTRATIONS
Particulate matter	< 20 mg/l
Total organic carbon	< 2 mg/l
Unionised ammonia	< 1 μg/l
Hardness as $CaCO_3$	< 400 mg/l*
Residual chlorine	< 10 μg/l
Total organophosphorus pesticides	< 50 ng/l
Total organochlorine pesticides plus polychlorinated biphenyls	< 50 ng/l
Total organic chlorine	< 25 ng/l

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

Appendix 5

GUIDANCE FOR TEST PERFORMANCE

Example of a breeding cage:

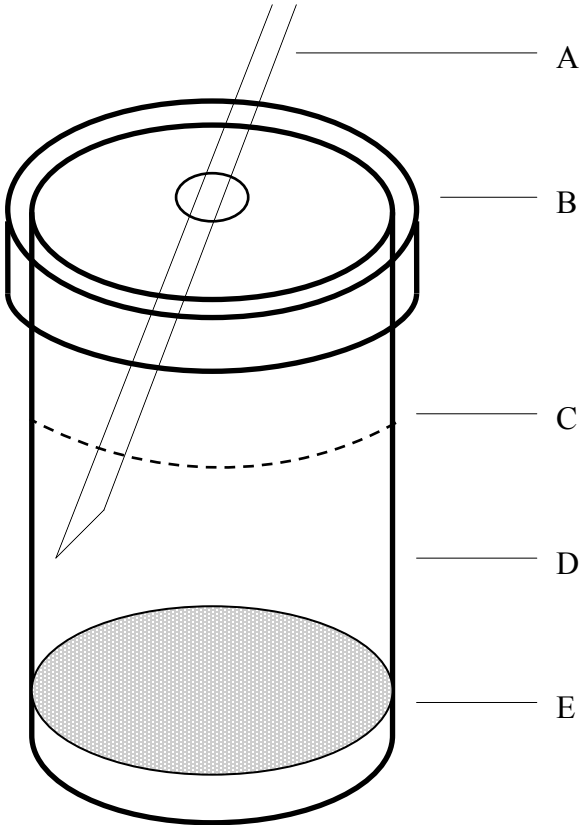


A: gauze on the top and at least one side of the cage (mesh size ca. 1 mm)

B: aperture for placing the emerged adults inside the breeding cage and to remove the laid egg ropes from the crystallisation dishes (not shown in this graphic)

C: breeding cage size minimum 30 cm length, 30 cm height and 30 cm width

Example of a test vessel:



A: pasteur pipette for air supply of the overlying water

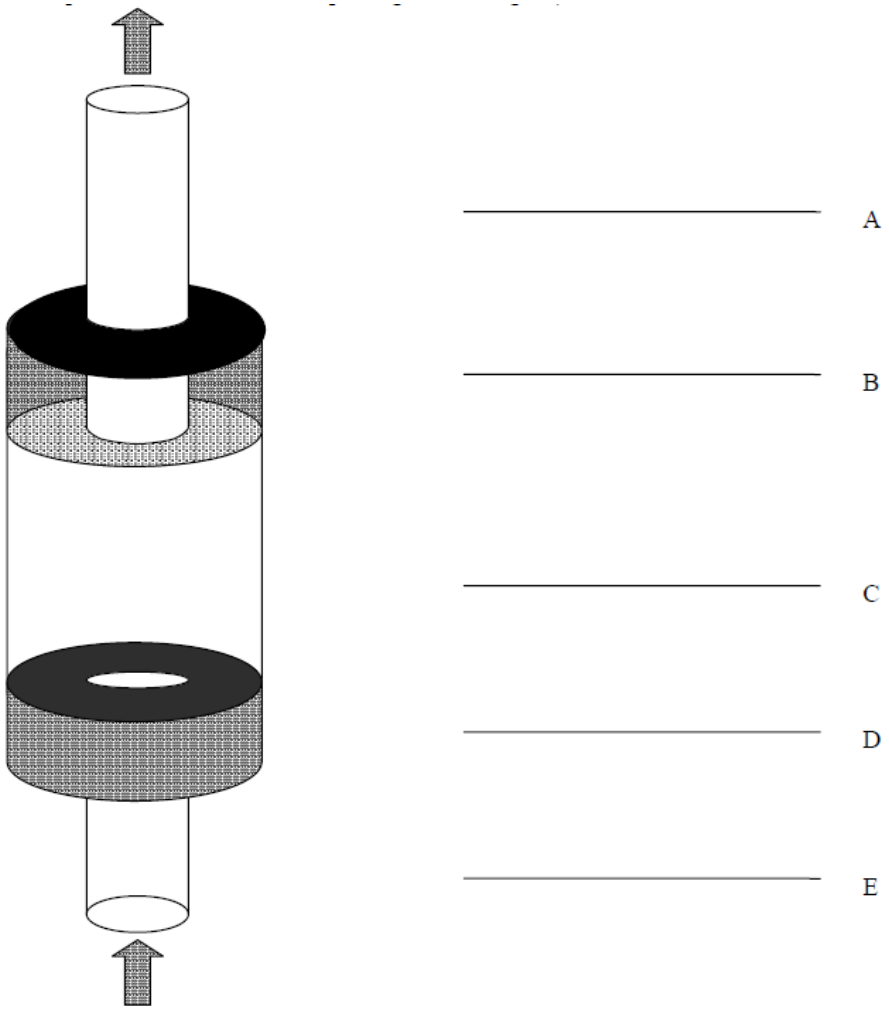
B: glass lid to prevent emerged midges from escaping

C: water surface layer

D: test vessel (glass beaker minimum 600 ml)

E: sediment layer

Example of an exhauster for capturing adult midges (arrows indicate air flow direction):



A: glass tube (inner diameter ca. 5 mm) connected to a self-priming pump

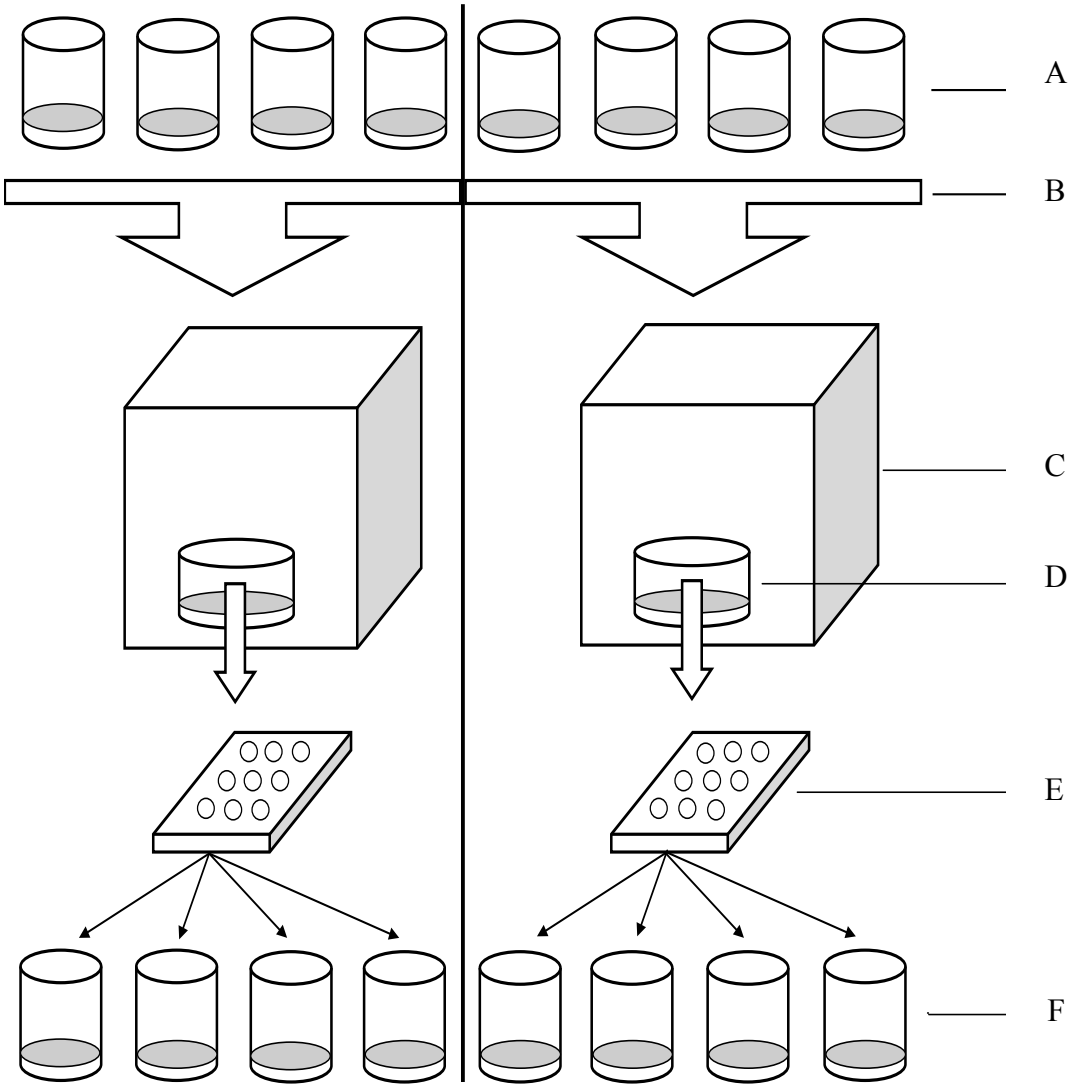
B: cork of vulcanised rubber, perforated with glass tube (A). On the inside, the opening of glass tube (A) is covered with some cotton and a gauze (mesh size ca. 1 mm) to prevent damaging the midges when they are sucked into the exhauster

C: transparent container (plastic or glass, length ca. 15 cm) for captured midges

D: cork of vulcanised rubber, perforated with tube (E). To release midges into the breeding cage, cork D is released from container C

E: tube (plastic or glass, inner diameter ca. 8 mm) to collect adult midges from vessel

Schematic presentation of a life-cycle test:



A: 1st generation – test vessels containing a sediment-water system, eight replicates, 20 first instar larvae per vessel

B: four test vessels for each breeding cage, A and B

C: breeding cages (A and B) for swarming, mating and oviposition

D: crystallising dishes for deposition of egg ropes

E: micro plates, one well for each egg rope

F: 2nd generation – test vessels containing a sediment-water system, eight replicates, 20 first instar larvae per vessel

C.41. Fish Sexual Development Test

INTRODUCTION

1. This test method is equivalent to OECD test guideline (TG) 234 (2011). It is based on a decision from 1998 to develop new or update existing test methods for the screening and testing of potential endocrine disrupters. The Fish Sexual Development Test (FSDT) was identified as a promising test method covering a sensitive fish life stage responsive to both oestrogen and androgen-like chemicals. The test method went through an inter-laboratory validation exercise from 2006 to 2010, where Japanese medaka (*Oryzias latipes*), zebrafish (*Danio rerio*) and three spined stickleback (*Gasterosteus aculeatus*) were validated and fathead minnow (*Pimephales promelas*) was partially validated (41) (42) (43). This protocol includes Japanese medaka, the three-spined stickleback and zebrafish. The protocol is in principle an enhancement of OECD TG 210 Fish, Early Life Stage Toxicity Test (1), where the exposure is continued until the fish are sexually differentiated, i.e. about 60 days post-hatch (dph) for Japanese medaka, the three-spined stickleback and zebrafish (the exposure period can be shorter or longer for other species that are validated in the future), and endocrine-sensitive endpoints are added. The FSDT assesses early life-stage effects and potential adverse consequences of putative endocrine disrupting chemicals (e.g. oestrogens, androgens and steroidogenesis inhibitors) on sexual development. The combination of the two core endocrine endpoints, vitellogenin (VTG) concentration and phenotypic sex ratio enable the test to indicate the mode of action of the test chemical. Due to the population-relevant change in phenotypic sex ratio, the FSDT can be used for hazard and risk assessment. However, if the test is used for hazard or risk assessment, the stickleback should not be used because the validation data available so far showed that in this species the alterations of phenotypic sex ratio by the test chemicals were uncommon.

2. The protocol is based on fish exposed via water to chemicals during the sex labile period in which the fish is expected to be most sensitive to the effects of endocrine disrupting chemicals that interfere with sexual development. Two core endpoints are measured as indicators of endocrine-associated developmental aberrations, the VTG concentrations and sex ratios (proportions of sex) determined via gonad histology. Gonadal histopathology (evaluation and staging of oocytes and spermatogenetic cells) is optional. Additionally, the genetic sex is determined whenever possible (e.g. in Japanese medaka and the three spined stickleback). The presence of a genetic sex marker is a considerable advantage as it increases the power of the sex ratio statistics and enables the detection of individual phenotypic sex reversal. Other apical endpoints that should be measured include hatching rate, survival, length and body weight. The test method might be adaptable to other species than those mentioned above provided that the other species undergo a validation equal to the one accomplished for Japanese medaka, the three-spined stickleback and zebrafish, that the control fish are sexually differentiated at the end of the test, that VTG levels are sufficiently high to detect significant chemical-related variations, and that sensitivity of the test system is established using endocrine active reference chemicals ((anti)-oestrogens, (anti)-androgens, aromatase inhibitors etc). In addition, any validation report(s) referring to FSDT data using other species should be reviewed by the OECD, and the validation

outcome should be considered as satisfactory.

Initial considerations and limitations

3. VTG is normally produced by the liver of female oviparous vertebrates in response to circulating endogenous oestrogen (2). It is a precursor of egg yolk proteins and, once produced in the liver, travels in the bloodstream to the ovary, where it is taken up and modified by developing eggs. The VTG synthesis is very limited, though detectable, in immature fish and adult male fish because they lack sufficient circulating oestrogen. However, the liver is capable of synthesising and secreting VTG in response to exogenous oestrogen stimulation (3) (4) (5).

4. The measurement of VTG serves for the detection of chemicals with oestrogenic, anti-oestrogenic, androgenic modes of action and chemicals that interfere with steroidogenesis as for example aromatase inhibitors. The detection of oestrogenic chemicals is possible via the measurement of VTG induction in male fish, and it has been abundantly documented in the scientific peer-reviewed literature. VTG induction has also been demonstrated following exposure to aromatisable androgens (6) (7). A reduction in the circulating level of oestrogen in females, for instance through the inhibition of the aromatase converting the endogenous androgen to the natural oestrogen 17β-oestradiol, causes a decrease in the VTG concentration, which is used to detect chemicals having aromatase inhibiting properties or steroidogenesis inhibitors more broadly (33). The biological relevance of the VTG response following oestrogenic/aromatase inhibition is established and has been broadly documented (8) (9). However, it is possible that production of VTG in females can also be affected by general toxicity and non-endocrine toxic modes of action.

5. Several measurement methods have been successfully developed and standardised for routine use to quantify VTG in blood, liver, whole body or head/tail homogenate samples collected from individual fish. This is the case for zebrafish, three-spined stickleback and Japanese medaka and also the partially validated species fathead minnow; species-specific Enzyme-Linked Immunosorbent Assay (ELISA) methods using immunochemistry for the quantification of VTG are available (5) (10) (11) (12) (13) (14) (15) (16) . In Japanese medaka and zebrafish, there is a good correlation between VTG measured from blood plasma, liver and homogenate samples although homogenates tend to show slightly lower values than plasma (17) (18) (19). Appendix 5 provides the recommended procedures for sample collection for VTG analysis.

6. Change in the phenotypic sex ratio (proportions of sex) is an endpoint reflecting sex reversal. In principle, oestrogens, anti-oestrogens, androgens, anti-androgens and steroidogenesis inhibiting chemicals can affect the sex ratio of developing fish (20). It has been shown that this sex reversal is partly reversible in zebrafish (21) following oestrogen-like chemical exposure, whereas sex reversal following androgen-like chemical exposure is permanent (30). The sex is defined as female, male, intersex (both oocytes and spermatogenetic cells in one gonad) or undifferentiated, determined in individual fish via histological examination of the gonads. Guidance is given in Appendix 7 and in the OECD Guidance Document on the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (22).

7. Genetic sex is examined via genetic markers when they exist in a given fish species. In Japanese medaka the female XX or male XY genes can be detected by Polymerase Chain-Reaction (PCR), or the Y-linked DM domain gene (DMY) can be analysed (DMY negative or positive) as described in (23) (24). In three-spined stickleback, there is an equivalent PCR method for genetic sex determination described in Appendix 10. Where the genetic sex can be individually linked to the phenotypic sex, the power of the test is improved and therefore genetic sex should be determined in species with documented genetic sex markers.

8. The two core endocrine endpoints, VTG and sex ratio, can in combination demonstrate the endocrine mode of action (MOA) of the chemical (Table 1). The sex ratio is a population relevant biomarker (25) (26) and for some well defined modes of action, the FSDT results may be used for hazard and risk assessment purposes when deemed appropriate by the regulatory agency. These modes of action are at present oestrogens, androgens and steroidogenesis inhibitors.

Table 1: Reaction of the endocrine endpoints to different modes of action of chemicals: **↑= increasing, ↓=decreasing, - =not investigated**

MOA	**VTG ♂**	**VTG ♀**	**Sex ratio**	**References**
Weak oestrogen agonist	↑	↑	↑♀ or ↑Undiff	(27) (40)
Strong oestrogen agonist	↑	↑	↑♀ or ↑Undiff, No ♂	(28) (40)
Oestrogen antagonist	-	-	↓♀, ↑Undiff.	(29)
Androgen agonist	↓ or -	↓ or -	↑ ♂, No ♀	(28) (30)
Androgen antagonist	-	-	↑♀ ↑Intersex	(31)
Aromatase inhibitor	↓	↓	↓♀	(33)

9. The FSDT does not cover the reproductive life stage of the fish and therefore chemicals that are suspected to affect reproduction at lower concentrations than sexual development should be examined in a test that covers reproduction.

10. Definitions for the purpose of this Test Method are given in Appendix 1.

11. The *in vivo* FSDT is intended to detect chemicals with androgenic and oestrogenic properties as well as anti-androgenic, anti-oestrogenic and steroidogenesis inhibiting properties. The FSDT validation phases (1 and 2) did cover oestrogenic, androgenic and steroidogenesis inhibiting chemicals. The effects in the FSDT of oestrogen- and androgen antagonists can be seen in Table 1 but these MOA are less documented at present time.

PRINCIPLE OF THE TEST

12. In the test, fish are exposed, from newly fertilised egg until the completion of sexual differentiation, to at least three concentrations of the test chemical dissolved in water. The test conditions should be flow-through unless not possible due to the availability or nature (e.g. limited solubility) of the test chemical. The test starts with the placing of newly fertilised eggs (before cleavage of the blastodisc) in the test chambers. The loading of the chambers is described for each species in paragraph 27. For the validated fish species, Japanese medaka, the three-spined stickleback and zebrafish, the test is terminated at 60 dph. At test termination, all fish are euthanised humanely. A biological sample (blood plasma, liver or head/tail homogenate) is collected for VTG analysis from each fish and the remaining part is fixed for histological evaluation of the gonads to determine the phenotypic sex; optionally, histopathology (e.g. staging of gonads, severity of intersex) can be performed. A biological sample (the anal- or the dorsal fin) for the determination of the genetic sex is taken in species possessing appropriate markers (Appendices 9 and 10).

13. An overview of relevant test conditions specific for validated species: Japanese medaka, the three-spined stickleback and zebrafish is provided in Appendix 2.

INFORMATION ON THE TEST CHEMICAL

14. Results from an acute toxicity test or other short-term toxicity assay [e.g. test method C.14 (34) and OECD TG 210 (1)], preferably performed with the species chosen for this test, should be available. This implies that the water solubility and the vapour pressure of the test chemical are known and a reliable analytical method for the quantification of the chemical in the test chambers, with known and reported accuracy and limit of detection, is available.

15. Other useful information includes the structural formula, purity of the chemical, stability in water and light, pKa, P_{ow} and results of a test for ready biodegradability (Test Method C.4) (35).

Test acceptance criteria

16. For the test results to be acceptable the following conditions apply:

- The dissolved oxygen concentration should be at least 60 per cent of the air saturation value (ASV) throughout the test;
- The water temperature should not differ by more than ± 1.5 °C between test chambers at any one time during the exposure period and be maintained within the temperature ranges specified for the test species (Appendix 2);
- A validated method for analysis of the exposure chemical with a detection limit well below the lowest nominal concentration should be available and evidence should be gathered to demonstrate that the concentrations of the test chemical in solution have been satisfactorily maintained within ±20% of the mean measured values;
- Overall survival of fertilised eggs in the controls and, where relevant, in the solvent controls, should be greater than or equal to the limits defined in Appendix 2;
- Acceptance criteria related to growth and proportions of sex at termination of the test are based on data from the control groups (pooled solvent and water control unless they are significantly different, then solvent only):

		Japanese medaka	Zebrafish	Three-spined stickleback
Growth	Fish wet weight, blotted dry	>150 mg	>75 mg	> 120 mg
	Length (standard length)	>20mm	>14 mm	>20 mm
Sex ratio (% males or females)		30-70 %	30-70 %	30-70%

- When a solvent is used it should have no statistical significant effect on survival and should not produce any endocrine disrupting effects or other adverse effects on the early-life stages as revealed by a solvent control.

If a deviation from the test acceptance criteria is observed, the consequences should be considered in relation to the reliability of the test data and these considerations should be included in the reporting.

DESCRIPTION OF THE TEST METHOD

Test chambers

17. Any glass, stainless steel or other chemically inert chambers can be used. The dimensions of the chambers should be large enough to allow compliance with loading rate criteria given below. It is desirable that test chambers be randomly positioned in the test area. A randomised block design with each concentration being present in each block is preferable to a completely randomised design. The test chambers should be shielded from unwanted disturbance.

Selection of species

18. Recommended fish species are given in Appendix 2. The procedures for inclusion of new species are given in paragraph 2.

Holding of parental fish

19. Details on holding the parental fish under satisfactory conditions may be found in OECD TG 210(1). Parental fish should be fed once or twice a day with appropriate food.

Handling of embryos and larvae

20. Initially, embryos and larvae may be exposed within a main chamber in smaller glass or stainless steel chambers, fitted with mesh sides or ends to permit a flow of test chemical through the chamber. Non-turbulent flow through these small chambers may be induced by suspending them from an arm arranged to move the chamber up and

down but always keeping the organisms submerged.

21. Where egg containers, grids or meshes have been used to hold eggs within the main test chamber, these restraints should be removed after the larvae hatch, except that meshes should be retained to prevent the escape of the fish. If there is a need to transfer the larvae, they should not be exposed to the air and nets should not be used to release fish from egg containers. The timing of this transfer varies with the species and transfer may not always be necessary.

Water

22. Any water in which the test species shows control survival at least as good as in water described in Appendix 3 is suitable as test water. It should be of constant quality during the period of the test. In order to ensure that the dilution water will not unduly influence the test result (for example by reacting with the test chemical) or adversely affect the performance of the brood stock, samples should be taken at intervals for analysis. Total organic carbon, conductivity, pH and suspended solids should be measured, for example every three months where dilution water is known to be relatively constant in quality. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g. Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Cl^{-}, SO_4^{2-}) and pesticides should be done, if water quality is questionable. Details about chemical analysis and water collection can be found in paragraph 34.

Test solutions

23. Flow-through system should be used if practically possible. For flow-through tests, a system that continually dispenses and dilutes a stock solution of the test chemical (e.g. metering pump, proportional diluter, and saturator system) is necessary to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and dilution water should be checked at intervals during the test and should not vary by more than 10% throughout the test. A flow rate equivalent to at least five test chamber volumes per 24 hours has been found suitable (1). Care should be taken to avoid the use of plastic tubing or other materials, some of which may contain biologically active chemicals or may adsorb the test chemical.

24. The stock solution should preferably be prepared without the use of solvents by simply mixing or agitating the test chemical in the dilution water by using mechanical means (e.g. stirring or ultrasonication). If the test chemical is difficult to dissolve in water, procedures described in the OECD Guidance Document on aquatic toxicity testing of difficult substances and mixtures should be followed (36). The use of solvents should be avoided but may be necessary in some cases in order to produce a suitably concentrated stock solution. Examples of suitable solvents are given in (36).

25. Semi-static test conditions should be avoided unless justification is provided on compelling reasons associated with the test chemical (e.g. stability, limited availability, high cost or hazard). For the semi-static technique, two different renewal procedures may be followed. Either new test solutions are prepared in clean chambers and surviving eggs and larvae gently transferred into the new chambers, or the test organisms are retained in the test chambers whilst a proportion (at least two thirds) of

the test water is changed daily.

PROCEDURE

Conditions of Exposure

Collection of eggs and duration

26. To avoid genetic bias, eggs are collected from a minimum of three breeding pairs or groups, mixed and randomly selected to initiate the test. For the three-spined stickleback, see the description of artificial fertilisation in Appendix 11. The test should start as soon as possible after the eggs have been fertilised, the embryos preferably being immersed in the test solutions before cleavage of the blastodisc commences, or as close as possible after this stage and no later than 12 h post fertilisation. The test should continue until sexual differentiation in the control group is completed (60 dph for Japanese medaka, the three-spined stickleback and zebrafish).

Loading

27. The number of fertilised eggs at the start of the test should be at least 120 per concentration divided between a minimum of 4 replicates (square root allocation to control is accepted). The eggs should be randomly distributed (by using statistical tables for randomisation) among treatments. The loading rate (for definition, see Appendix 1) should be low enough in order that a dissolved oxygen concentration of at least 60% of the ASV can be maintained without direct aeration of the chambers. For flow-through tests, a loading rate not exceeding 0.5 g/l per 24 hours, and not exceeding 5 g/l of solution at any time is recommended. No later than 28 days post fertilisation the number of fish per replicate should be redistributed, so that each replicate contains as equal a number of fish as possible. If exposure related mortality occurs, the number of replicates should be reduced appropriately so that fish density between treatment levels is kept as equal as possible.

Light and temperature

28. The photoperiod and water temperature should be appropriate for the test species (see Appendix 2 for experimental conditions for the FSDT).

Feeding

29. Food and feeding are critical, and it is essential that the correct food for each stage is supplied at appropriate time intervals and at a level sufficient to support normal growth. Feeding should be *ad libitum* whilst minimising the surplus. To obtain a sufficient growth rate, fish should be fed at least twice daily (accepting once daily on weekends), separated by at least three hours between each feed. Surplus food and faeces should be removed, as necessary, to avoid accumulation of waste. As experience is gained, food and feeding regimes are continuously being refined to improve survival and optimise growth. Effort should therefore be made to confirm the proposed regime with acknowledged experts. Feeding should be withheld 24 hours before ending the test. Examples of appropriate food items are listed in Appendix 2 (see also the OECD

Fish Testing Framework (39).

Test concentrations

30. Test chemicals should be spaced as described in Appendix 4. A minimum of three test concentrations in at least four replicates should be used. The curve relating LC_{50} to period of exposure in the acute studies available should be considered when selecting the range of test concentrations. Five test concentrations are recommended if the data are to be used for risk assessment.

31. Concentrations of the chemical higher than 10% of the acute adult LC_{50} or 10 mg/l, whichever is the lower, need not be tested. The maximum test concentration should be 10% of the LC_{50} on the larval/juvenile life-stage.

Controls

32. A dilution water control (≥ 4 replicates) and, if relevant, a solvent control (≥ 4 replicates) should be run in addition to the test concentrations. Only solvents that have been investigated not to have any statistical significant influence on the test endpoints should be used in the test.

33. Where a solvent is used, its final concentration should not be greater than 0.1 ml/l (36) and it should be the same concentration in all test chambers, except the dilution water control. However, every effort should be made to avoid the use of such solvent or keep solvent's concentrations to a minimum.

Frequency of Analytical Determinations and Measurements

34. Chemical analysis of the test chemical concentration should be performed before initiation of the test to check compliance with the acceptance criteria. All replicates should be analysed individually at the beginning and termination of the test. One replicate per test concentration should be analysed at least once per week during the test, changing systematically between replicates (1,2,3,4,1,2….). If samples are stored to be analysed at a later time, the storage method of the samples should be previously validated. Samples should be filtered (e.g. using a 0.45 μm pore size) or centrifuged to ensure that the determinations are made on the chemical in true solution.

35. During the test, dissolved oxygen, pH, total hardness, conductivity, salinity (if relevant), and temperature should be measured in all test chambers. As a minimum dissolved oxygen, salinity (if relevant), and temperature should be measured weekly, and pH, conductivity and hardness at the beginning and at the end of the test. Temperature should preferably be monitored continuously in at least one test chamber.

36. Results should be based on measured concentrations. However, if the concentration of the test chemical in solution has been satisfactorily maintained within ±20% of the nominal concentration throughout the test, then the results can either be based on nominal or measured values.

Observations and measurements

Stage of embryonic development

37. The exposure should begin as soon as possible after fertilisation and before cleavage of the blastodisc commences and no later than 12 h post fertilisation to ensure exposure during early embryonic development.

Hatching and survival

38. Observations on hatching and survival should be made at least once daily and numbers recorded. Dead embryos, larvae and juvenile fish should be removed as soon as observed since they can decompose rapidly and may be broken up by the actions of the other fish. Extreme care should be taken when removing dead individuals not to knock or physically damage adjacent eggs/larvae, these being extremely delicate and sensitive. Criteria for death vary according to life stage:

- for eggs: particularly in the early stages, a marked loss of translucency and change in coloration, caused by coagulation and/or precipitation of protein, leading to a white opaque appearance;
- for larvae and juvenile fish: immobility and/or absence of respiratory movement and/or absence of heart-beat and/or white opaque coloration of central nervous system and/or lack of reaction to mechanical stimulus.

Abnormal appearance

39. The number of larvae or fish showing abnormality of body form should be recorded, and the appearance and the nature of the abnormality described. It should be noted that abnormal embryos and larvae occur naturally and can be of the order of several per cent in the control(s) in some species. Abnormal animals should only be removed from the test chambers on death. However, in accordance with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, if abnormalities result in pain, suffering and distress or lasting harm, and death can be reliably predicted, animals should be anaesthetised and euthanised according to the description in paragraph 44 and treated as mortality for data analysis..

Abnormal behaviour

40. Abnormalities, e.g. hyperventilation, uncoordinated swimming, atypical quiescence and atypical feeding behaviour should be recorded at appearance.

Weight

41. At the end of the test all surviving fish should be euthanised (anaesthetised if blood samples should be taken), and individual wet weight (blotted dry) should be measured.

Length

42. At the end of the test, individual lengths (standard length) should be measured.

43. These observations will result in some or all of the following data being available for reporting:

- cumulative mortality;
- numbers of healthy fish at end of test;
- time to start of hatching and end of hatching;
- length and weight of surviving animals;
- numbers of deformed larvae;
- numbers of fish exhibiting abnormal behaviour.

Sampling of fish

44. Fish sampling is performed at termination of the test. Sampled fish should be euthanised with e.g. MS-222 (100-500 mg per l buffered with 200 mg $NaHCO_3$ per l) or FA-100 (4-allyl-2-methoxyphenol: eugenol) and individually measured and weighed as wet weight (blotted dry) or anaesthetised if a blood sample should be taken (see paragraph 49).

Sampling for VTG analysis and sex determination via histological evaluation

45. All fish should be sampled and prepared for analysis of sex and VTG. All fish should be analysed histologically to determine sex. For the VTG measurements, a sub-sampling of at least 16 fish from each replicate is accepted. More fish should be analysed for VTG if the results of the sub-sampling turn out to be unclear.

46. The sampling procedure for VTG and sex determination is dependent on the VTG analysis method:

Head/tail homogenate method for VTG analysis

47. The fish is euthanised. Head and tail of each fish are separated from the body of the fish by cuts made right behind the pectoral fins, and right behind the dorsal fin, using a scalpel (See Figure 1). The head and tail part from each fish are pooled, weighed and individually numbered, frozen in liquid nitrogen and stored at -70º or less for VTG analysis. The body part of the fish is numbered and fixed in an appropriate fixative for histological evaluation (22). By use of this method VTG and histopathology are evaluated on each individual and a possible change in the VTG level can thus be related to the phenotypic sex of the fish or genetic sex (Japanese medaka and the three-spined stickleback) of the fish. For further information see guidance for homogenisation (Appendix 5) and guidance for VTG quantification (Appendix 6).

Liver homogenate method for VTG analysis

48. The fish is euthanised. The liver is dissected out and stored at -70º C or below. Recommended procedures for liver excision and pre-treatment are available in OECD TG 229 (37) or Chapter C.37 of this Annex (38). Livers are then individually homogenised as described in OECD TG 229 or Chapter C.37 of this Annex. The supernatant collected is used for measuring VTG with a homologous ELISA technique

(see Appendix 6 for an example of quantification in zebrafish or OECD TG 229 (37) for Japanese medaka). Following this approach, it is also possible to have individual fish data on both VTG and gonad histology.

Blood plasma method for VTG analysis

49. Blood is collected from the anaesthetised fish by cardiac puncture, caudal vein or tail cutting, and centrifuged at 4º C for plasma collection. The plasma is stored at -70º C or below until use. The whole fish is euthanised and fixed for histology. Both plasma samples and fish are numbered individually to relate VTG levels to the sex of the fish.

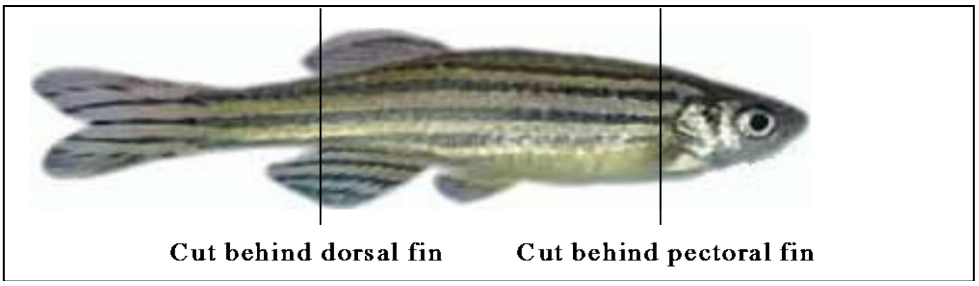


Figure 1: How to cut a fish for measurement of VTG in head/tail homogenate and histological evaluation of the mid section

Genetic sex determination

50. A biological sample for the determination of the genetic sex is taken from individual fish in species possessing appropriate markers. For Japanese medaka, the anal fin or dorsal fin is collected. A detailed description is given in Appendix 9 including tissue sampling and sex determination by a PCR-method. Equally, for the three spined stickleback, a description of tissue sampling and a sex determining PCR-method is given in Appendix 10.

VTG measurement

51. The measurement of VTG should be based upon a quantitative and analytically validated method. Information should be available upon the intra-assay and inter-assay variability of the method used in a given laboratory. The source of inter- and intra-laboratory variability is (most likely) based on the different developing stages of the fish population. Considering the variability of VTG measurement, NOECs based on this endpoint alone should be treated with great care. Different methods are available to assess VTG production in the fish species considered in this assay. A measurement technique that is both relatively sensitive and specific is the determination of protein concentrations via enzyme-linked immunosorbent assay (ELISA). Homologous antibodies (raised against VTG of the same species) and most important homologous standards should be used.

Sex determination

52. Dependent on the VTG sampling procedure, whole fish or the remaining mid-section of each fish is placed in a pre-labelled processing cassette and fixed in an appropriate fixative for histological determination of sex (optionally also for evaluation of gonadal staging). Guidance on fixation and embedding is provided in Appendix 7 as well as in the OECD Guidance Document on the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (22). After processing, the fish are embedded in paraffin blocks. The individuals should be placed longitudinally in the paraffin block. At least six longitudinal sections (3-5 μm in thickness) in a frontal plane including gonadal tissue from both gonads are taken from each individual. The interval between these sections should be approximately 50 μm for males and 250 μm for females. However, since each block will often contain males and females (if more than one individual are embedded in each block), the interval between sections from these blocks should be approximately 50 μm until at least six sections of the gonads from each male are obtained. Thereafter, the interval between sections can be increased to approximately 250 μm for the females. Sections are stained with haematoxylin and eosin and examined by light-microscopy with focus on sex (male, female, intersex or undifferentiated). Intersex is defined as presence of more than one oocyte in testis per six sections analysed or spermatogenic cells (yes/no) in ovaries. Histopathology and staging of ovaries and testis is optional but if investigated, the results should be statistically analyzed and reported. It should be noted that some fish species naturally lack a fully developed pair of gonads and only one gonad may be present (e.g. Japanese medaka and occasionally zebrafish). All such observations should be recorded.

53. Genetic sex determination in individual Japanese medaka is based on the presence or absence of the medaka male-sex determining gene, DMY, which is located on the Y chromosome. The genotypic sex of medaka can be identified by sequencing the DMY gene from DNA extracted from for instance a piece of anal fin or dorsal fin. The presence of DMY indicates a XY (male) individual regardless of phenotype, while the absence of DMY indicates a XX (female) individual regardless of phenotype (23). Guidance for tissue preparation and PCR method is given in Appendix 9. The genetic sex determination in individual three-spined stickleback is also performed via a PCR method, described in Appendix 10.

54. The occurrence of intersex (for definition, see Appendix 1) should be reported.

Secondary sexual characteristics

55. Secondary sexual characteristics are under endocrine control in species like the Japanese medaka; therefore observations of physical appearance of the fish should if possible be made at the end of the exposure. In the Japanese medaka, the papillary formation on the posterior part of the anal fin in females is androgen sensitive. Chapter C.37 of this Annex (38) provides relevant photographs of male secondary sex characteristics and androgenised females.

DATA AND REPORTING

Treatment of results

56. It is important that the strongest valid statistical test determine the endpoint. The replicate is the experimental unit but intra-replicate variability should be included in the statistical testing. A decision flow-chart is available in Appendix 8 to help with the most appropriate statistical test to use based on the characteristic of the data obtained from the test. Statistical significance level is 0.05 for all endpoints included.

Proportions of sex and genetic sex

57. The proportions of sex should be analysed for significant effect (NOEC/LOEC approach) of exposure by Jonckheere-Terpstra (Trend test) if a monotone dose-response exists. If non-monotonicity is found then a pair wise test should be applied: Use Dunnett´s test if normality and homogenous variance can be obtained. Use Tamhane-Dunnett if heterogeneous variance is present. Otherwise use exact Mann-Whitney test with Bonferroni-Holm adjustment. A flow chart describing the statistics of the proportions of sex is placed in Appendix 8. The proportions of sex should be presented in tables as concentration proportions ± SD of males, females, intersex and undifferentiated. Statistical significance should be highlighted. Examples are presented in the FSDT Phase 2 validation report (42). Genetic sex should be reported as percentage of phenotypic sex reversal of males, females, intersex and undifferentiated.

VTG concentrations

58. VTG concentrations should be analysed for significant effect (NOEC/LOEC approach) of exposure. The Dunnett test is preferable to the t-test with Bonferroni correction. Where a Bonferroni correction is used, the Bonferroni-Holm correction is preferable. Allowance should be made for log-transformation of VTG to achieve normality and variance homogeneity. Next, if the concentration-response is consistent with monotonicity, then the JonckheereTerpstra test is preferable to any of the above. If t-tests or Dunnett's test is used, there is no need for a ANOVA significance F-test in order to proceed. For details see the flow chart in Appendix 8. Results should be reported in tables as concentration means ± SD for males, females, intersex and undifferentiated separately. Statistical significance for phenotypic females and phenotypic males should be highlighted. Examples are presented in the FSDT Phase 2 validation report (42).

Test chemical actual concentrations

59. The actual chamber concentrations of the test chemical should be analysed in frequencies described in paragraph 34. Results should be reported in tables as mean concentration ± SD on replicate basis as well as on concentration basis with information on number of samples and with outliers from the mean treatment concentration ± 20% highlighted. Examples can be found in the FSDT Phase 2 validation report (42).

Interpretation of results

60. The test results should be interpreted with caution where measured test chemical concentrations in test solutions occur at levels near the detection limit of the analytical

method.

Test report

61. The test report should include the following information:

Test chemical

- Relevant physical-chemical properties; chemical identification data including purity and analytical method for quantification of the test chemical.

Test conditions

- Test procedure used (e.g. flow-through semi-static/renewal); test design including test concentrations, method of preparation of stock solutions (in an Annex), frequency of renewal (the solubilising agent and its concentration should be given, when used);
- The nominal test concentrations, the means of the measured values and their standard deviations in the test chambers and the method by which these were attained (the analytical method used should be presented in an Annex);Evidence that the measurements refer to the concentrations of the test chemical in true solution;
- Water quality within test chambers: pH, hardness, temperature and dissolved oxygen concentration;
- Detailed information on feeding (e.g. type of food(s), source, amount given and frequency and analyses for contaminants (e.g. PCBs, PAHs and organochlorine pesticides) if relevant.

Results

- Evidence that controls met the validity criteria: data on hatching rate should be presented in tables as percentage per replicate and per concentration. Outliers from the acceptance criteria (in controls) should be highlighted. Survival should be presented as percentage per replicate and per concentration. Outliers from the validity criteria (in controls) should be highlighted;
- Clear indication of the results obtained on the different endpoints observed: embryo survival and hatching success; external abnormalities; length and weight; VTG measurements (ng/g homogenate, ng/ml plasma or ng/mg liver); gonadal histology, sex ratio, genetic sex data; incidence of any unusual reactions by the fish and any visible effects produced by the test chemical.

62. The results should be presented as mean values ± standard deviation (SD) or standard error (SE). Statistics should be reported as a minimum as NOEC and LOEC and confidence intervals. The statistical flow chart (Appendix 8) should be followed.

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Appendix 1

ABBREVIATIONS & DEFINITIONS

Apical endpoint: Causing effect at population level

ASV: Air saturation value

Biomarker: Causing effect at individual level

Chemical: A substance or a mixture.

Dph: Days post hatch

DMY: Y-specific DM-domain gene required for male development in the medaka fish

ELISA: Enzyme-Linked Immunosorbent Assay

Fish weight: Fish wet weight (blotted dry)

FSDT: Fish Sexual Development Test

HPG axis: Hypothalamic-pituitary-gonadal axis

Intersex fish: Fish with more than one oocyte in testis per 6 sections analysed or spermatogenetic cells in ovaries (yes/no)

Loading rate: Wet weight of fish per volume of water

MOA: Mode of action

RT-PCR: Reverse Transcriptase Polymerase Chain-Reaction

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

Undifferentiated fish: Fish with gonads exhibiting no discernible germ cells.

VTG: Vitellogenin

Appendix 2

EXPERIMENTAL CONDITIONS FOR THE FSDT (FRESHWATER SPECIES)

1.Recommended species	Japanese medaka (*Oryzias latipes*)	Zebrafish (*Danio rerio*)	Three-spined Stickleback (*Gasterostreus aculeatus*)
2. Test type	Flow-through or semi-static	Flow-through or semi-static	Flow-through or semi-static
3. Water temperature	25± 2°C	27 ± 2°C	20± 2°C
4. Illumination quality	Fluorescent bulbs (wide spectrum)	Fluorescent bulbs (wide spectrum)	Fluorescent bulbs (wide spectrum
5. Light intensity	10-20 µE/m^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)	10-20 µE/m^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)	10-20 µE/m^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)
6. Photoperiod	12-16 h light, 8-12 h dark	12-16 h light, 8-12 h dark	16 h light, 8 h dark
7. Minimum chamber size	Individual chambers should contain a minimum of 7 l water volume	Individual chambers should contain a minimum of 7 l water volume	Individual chambers should contain a minimum of 7 l water volume
8. Volume exchanges of test solutions	Minimum of 5 daily	Minimum of 5 daily	Minimum of 5 daily

9. Age of test organisms at start of exposure	Newly fertilised eggs (Early blastula stage)	Newly fertilised eggs (Early blastula stage)	Newly fertilised eggs
10. No. of eggs per treatment	Minimum 120	Minimum 120	Minimum 120
11. No. of treatments	Minimum 3 (plus appropriate controls)	Minimum 3 (plus appropriate controls)	Minimum 3 (plus appropriate controls)
12. No. replicates per treatment	Minimum 4 (unless square root allocation to controls)	Minimum 4 (unless square root allocation to controls)	Minimum 4 (unless square root allocation to controls)
13. Feeding regime	Live *Artemia*, frozen adult brine shrimp, flake food, etc. It is recommended to feed twice daily	Special fry food, live *Artemia*, frozen adult brine shrimp, flake food, etc. It is recommended to feed twice daily	Live *Artemia*, frozen adult brine shrimp, flake food, etc. It is recommended to feed twice daily
14. Aeration	None unless DO concentration falls below 60% saturation	None unless DO concentration falls below 60% saturation	None unless DO concentration falls below 70% saturation
15. Dilution water	Clean surface, well or reconstituted water	Clean surface, well or reconstituted water	Clean surface, well or reconstituted water
16. Test chemical exposure duration	60-dph	60-dph	60-dph
17. Biological endpoints	Hatching success, Survival Gross-morphology, VTG gonadal histology,	Hatching success, Survival Gross- morphology, VTG gonadal histology, Sex	Hatching success, Survival Gross- morphology, VTG

	Genetic sex Sex ratio	ratio	gonadal histology, Sex ratio
18. Test acceptability criteria for pooled replicates of controls	Hatching success > 80% Post hatch survival ≥ 70% growth (Fish wet weight, blotted dry) > 150 mg Length (standard length) >20mm Sex ratio (% males or females) 30%-70%	Hatching success > 80% Post hatch survival ≥ 70% growth (Fish wet weight, blotted dry) > 75 mg Length (standard length) >14 mm Sex ratio (% males or females) 30%-70%	Hatching success > 80% Post hatch survival ≥ 70% growth (Fish wet weight, blotted dry) > 120 mg Length (standard length) >20 mm Sex ratio (% males or females) 30%-70%

Appendix 3

CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

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

Appendix 4

FROM TEST METHOD C.14 /GUIDANCE ON TEST CONCENTRATIONS

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

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

Appendix 5

GUIDANCE FOR HOMOGENISATION OF HEAD & TAIL FROM JUVENILE ZEBRAFISH, FATHEAD MINNOW, THREE SPINED STICKLEBACK AND JAPANESE MEDAKA

The purpose of this section is to describe the procedures that occur prior to the quantification of the VTG concentration. Other procedures that result in comparable VTG quantification can be used. It is an option to determine the VTG concentration in blood plasma or liver instead of head/tail homogenate.

Procedure

1. The fish are anaesthetised and euthanised in accordance with the test description.

2. The head and tail are cut of the fish in accordance with the test description. **Important**: All dissection instruments, and the cutting board should be rinsed and cleaned properly (e.g. with 96% ethanol) between handling of each single fish to prevent "VTG pollution" from females or induced males to un-induced males.

3. The weight of the pooled head and tail from each fish is measured to the nearest mg.

4. After being weighed, the parts are placed in appropriate tubes (e.g. 1.5 ml eppendorf) and frozen at –80 ºC until homogenisation or directly homogenised on ice with two plastic pistils. (Other methods can be used if they are performed on ice and the result is a homogenous mass). **Important**: *The tubes should be numbered properly so that the head and tail from the fish can be related to their respective body-section used for gonad histology.*

5. When a homogenous mass is achieved an amount of 4-10 time the tissue weight of ice-cold **homogenisation buffer*** is added (note the dilution). Keep working with the pistils until the mixture is homogeneous. **Important note**: *New pistils are used for each fish.*

6. The samples are placed on ice until centrifugation at **4°C** at 50000 g for 30 min.

7. Use a pipette to dispense portions of 20 to 50 µl (note the amount) supernatant into **at least two** tubes by dipping the tip of the pipette below the fat layer on the surface and carefully sucking up the supernatant without fat- or pellet fractions.

8. The tubes are stored at -80°C until use.

**Homogenisation buffer:*

50 mM Tris-HCl pH 7,4; 1% Protease inhibitor cocktail (Sigma): 12 ml Tris-HCl pH 7,4 + 120 µl Protease inhibitor cocktail (or equivalent protease inhibitor cocktails).

TRIS: TRIS-ULTRA PURE (ICN)

Protease inhibitor cocktail: From Sigma (for mammalian tissue) Product number **P 8340.**

NOTE: The homogenisation buffer should be used the same day as manufactured. Place on ice during use

APPENDIX 6

GUIDANCE FOR QUANTIFICATION OF HEAD & TAIL HOMOGENATE VITELLOGENIN IN ZEBRAFISH (DANIO RERIO) (MODIFIED FROM HOLBECH ET AL., 2001). OTHER PROCEDURES USING HOMOLOGOUS ANTIBODIES AND STANDARDS CAN BE USED

1. Microtiter plates (certified Maxisorp F96, Nunc, Roskilde Denmark) previously coated with 5 µg/ml anti zebrafish lipovitellin-IgG are thawed and washed 3 times with washing buffer*.

2. Purified zebrafish vitellogenin standard[1] is serially diluted to 0.2, 0.5, 1, 2, 5, 10 and 20 ng/ml in dilution buffer** and samples are diluted at least 200 times (to prevent matrix effect) in dilution buffer and applied to the plates. An assay control is applied in duplicate. 150 µl are applied to each well. Standards are applied in duplicate and samples in triplicate. Incubate over night at 4°C on a shaker.

3. The plates are washed 5 times with washing buffer*

4. HRP coupled to a dextran chain (e.g. AMDEX A/S, Denmark) and conjugated antibodies are diluted in washing buffer; Actual dilution differs by batch and age. 150 µl are applied to each well and the plates are incubated for 1 hour at room temperature on a shaker.

5. The plates are washed 5 times with washing buffer* and the bottom of the plates is carefully cleaned with ethanol.

6. 150 µl TMB plus*** are applied to each well. Protect the plate against light with tinfoil, and watch the colour development on a shaker.

7. When the standard curve is fully developed the enzyme activity is stopped by adding 150 µl 0.2 M H_2SO_4 to each well.

8. The absorbance is measured at 450 nm (e.g. on a Molecular Devices Thermomax plate reader). Data are analysed on the associated software (e.g. Softmax).

***Washing buffer:**

PBS-stock**** 500.0 ml

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[1] Battelle AP4.6.04 (1.18 mg/ml (AAA)), purified according to: Denslow, N.D., Chow, M.C., Kroll, K.J., Green, L. (1999). Vitellogenin as a biomarker of exposure for estrogen or estrogen mimics. Ecotoxicology 8: 385-398.

BSA	5.0	g
Tween 20	5.0	ml

Adjust pH to 7.3 and fill to 5 l with millipore H_2O. Store at 4º C.

****Dilution buffer:**

PBS-Stock****	100.0	ml
BSA	3.0	g
Tween 20	1.0	ml

Adjust pH to 7.3 and fill to 1 l with millipore H_2O. Store at 4º C.

*** TMB plus is a ”ready-to-use” substrate produced by KemEnTec (Denmark). It is sensitive to light. Store at 4º C.

******PBS stock**

NaCl	160.0	g
KH_2PO_4	4.0	g
$Na_2HPO_4 \cdot 2H_2O$	26.6	g
KCl	4.0	g

Adjust pH to 6.8 and fill with millipore H_2O to 2 l. Store at room temperature.

APPENDIX 7

GUIDANCE FOR THE PREPARATION OF TISSUE SECTIONS FOR SEX DETERMINATION AND STAGING OF GONADS

The purpose of this section is to describe the procedures that occur prior to the evaluation of histological sections. Other procedures that result in similar sex determination and gonadal staging can be used.

With a few exceptions, these procedures are similar for Japanese medaka (JMD) and zebrafish (ZF).

Euthanasia, Necropsy, and Tissue Fixation

Objectives:

1. Provide for the humane sacrifice of fish.

2. Obtain necessary body weights and measurements.

3. Evaluate secondary sex characteristics.

4. Dissect tissues for VTG analysis.

5. Fixation of the gonads.

Procedures:

1. Fish should be sacrificed immediately prior to necropsy. Therefore, unless multiple prosectors are available, multiple fish should not be sacrificed simultaneously.

2. Using the small dip net, a fish is removed from the experimental chamber and transported to the necropsy area in the transport container.

3. The fish is placed in the euthanasia solution. The fish is removed from the solution when there is cessation of respiration and the fish is unresponsive to external stimuli.

4. The fish is wet weighed.

5. For preparation of tissues for VTG analysis, the fish can be placed on a corkboard on the stage of a dissecting microscope.

    a. For zebrafish the head is cut right behind the pectoral fin and tail is cut right behind the dorsal fin.

    b. For Japanese medaka the abdomen is opened via a carefully made incision that

extends along the ventral midline from the pectoral girdle to a point just cranial to the anus. Using the small forceps and small scissors, the liver is carefully removed.

6. Specimen for VTG analysis are placed in eppendorf tubes and immediately frozen in liquid nitrogen.

7. The carcass including the gonads is placed into a pre-labelled plastic tissue cassette, which is transferred into Davidson's or Bouin´s fixative. The volume of fixative should be at least 10 times the approximated volume of the tissues. The fixative container is gently agitated for five seconds to dislodge air bubbles from the cassette.

8. a. All tissues remain in Davidson's fixative overnight, followed by transfer to individual containers of 10 % neutral buffered formalin the next day. Containers with cassettes are gently agitated for 5 seconds to ensure adequate penetration of formalin into cassettes.

b. Tissues remain in Bouins fixative for 24 h, followed by transfer to 70 % ethanol.

Tissue Processing

Objectives:

1. Dehydrate tissue for adequate penetration of paraffin.

2. Impregnate the tissue with paraffin to maintain tissue integrity and create a firm surface for microtomy.

Procedures:

3. Labelled tissue cassettes are removed from formalin/ethanol storage and the cassettes are placed in the processing basket(s). The processing basket is loaded in the tissue processor.

4. The processing schedule is selected.

5. After the tissue processor has completed the processing cycle, the basket(s) may be transferred to the embedded station.

Embedding

Objective:

Properly orient the specimen in solidified paraffin for microtomy.

Procedures:

1. The basket(s) of cassettes is/are removed from the processor and immersed in the paraffin-filled front chamber of the embedding station thermal console or the cassettes are moved to a separate paraffin heater.

2. The first cassette to be embedded is removed from the front chamber of the thermal console or the paraffin heater. The cassette lid is removed and discarded, and the cassette label is checked against the animal records to resolve potential discrepancies prior to embedding.

3. An appropriately sized embedding mould is selected.

4. The mould is held under the spout of the dispensing console and filled with molten paraffin.

5. The specimen is removed from the cassette and placed in the molten paraffin in the mould. This is repeated with 4-8 specimens for each paraffin mould. The position of individual fish is marked by putting fish no 1 in 180 degrees to fish 2-4/8.

6. Additional paraffin is added to cover the specimen.

7. The mould with the cassette base is placed on the cooling plate of the cryo console.

8. After the paraffin has solidified, the block (i.e., the hardened paraffin containing the tissues and the cassette base) is removed from the mould.

Microtomy

Objective:

Cut and mount histological sections for staining.

Procedures:

1. The initial phase of microtomy termed “facing” is conducted as follows:

    a. The paraffin block is placed in the chuck of the microtome.

    b. The chuck is advanced by rotating the microtome wheel and thick sections are cut from the paraffin surface of the block until the knife reaches the embedded tissues.

    c. The section thickness on the microtome is set between 3 – 5 microns. The chuck is advanced and multiple sections are cut from the block to remove any artefacts created on the cut surface of the tissue during rough trimming.

    d. The block can be removed from the chuck and placed facedown on ice to soak the tissue.

2. The next phase of microtomy is final sectioning and mounting of tissue sections on slides. These procedures are conducted as follows:

    a. If the block has been placed on ice, the block is removed from the ice and replaced in the chuck of the microtome.

b. With the section thickness on the microtome set to 3 – 5 microns, the chuck is advanced by rotating the microtome wheel. Sections are cut from the block until a "ribbon" containing at least one acceptable section including the gonads has been produced. (As necessary during sectioning, the block may be removed from the chuck, placed on ice to soak the tissue, and replaced in the chuck.)

c. The sections are floated flat on the surface of the water in the water bath. An attempt is made to obtain at least one section that contains no wrinkles and has no air bubbles trapped beneath it.

d. A microscope slide is immersed beneath the best section, which is lifted out of the water using the slide. This process is referred to as "mounting" the section on the slide.

e. Three sections are prepared for a set of fish. The second and third sections are taken at 50 micron intervals following the first section. If the fish are not embedded with their gonads in the same sectioning level, more sections are to be made to ensure that at least six sections including the gonads are obtained from each fish.

f. With a slide-marking pen, the block number from which the slide was produced is recorded on the slide.

g. The slide is placed in a staining rack.

h. The block is removed from the chuck and placed facedown for storage.

Staining, Cover slipping, and Slide Labelling

Objectives:

- Stain the sections for histopathological examination
- Permanently seal mounted and stained tissues.
- Permanently identify stained sections in a manner that allows complete traceability.

Procedures:

1. Staining

   a. Slides are air-dried overnight before staining.

   b. The sections are stained by Hematoxylin-Eosin.

2. Cover slipping

   a. Cover slips can be applied manually or automatically.

   b. A slide is dipped in xylene or TissueClear, and the excess

xylene/TissueClear is gently knocked off the slide.

c. Approximately 0.1 ml of mounting medium is applied near the end of the slide opposite to the frosted end or on the cover slip.

d. The cover slip is tilted at a shallow angle as it is applied to the slide.

3. Labelling

a. Each slide label should contain the following information.

i. Laboratory name

ii. Species

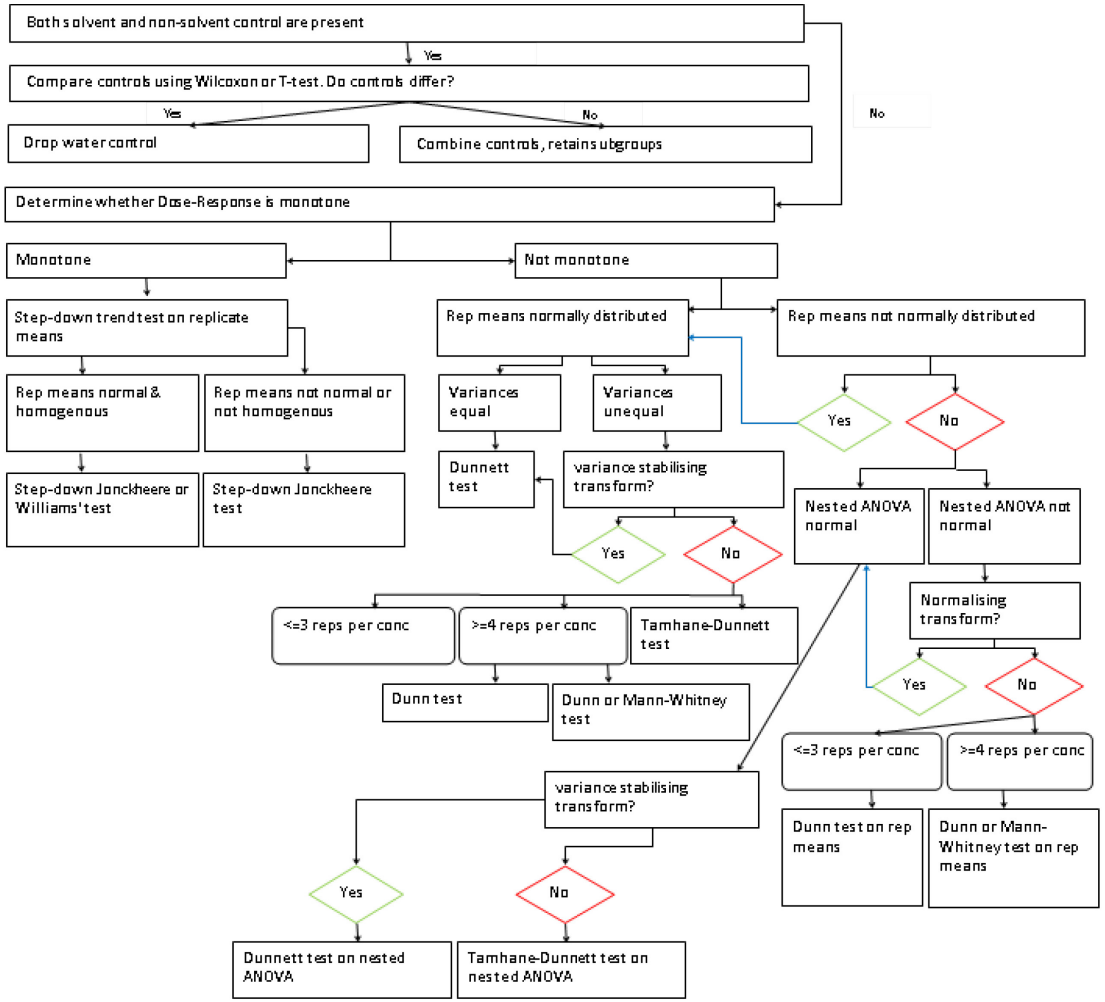
iii. Specimen No. / Slide No.

iv. Chemical / Treatment group

v. Date

APPENDIX 8

STATISTICAL FLOW CHART FOR VITELLOGENIN ANALYSIS


Both solvent and non-solvent control are present
Yes
Compare controls using Wilcoxon or T-test. Do controls differ?
Yes
No
Drop water control
Combine controls, retains ubgroups
No
Determine whether Dose-Response is monotone
Monotone
Not monotone
Step-down trend test on replicate means
Rep means normal & homogenous
Rep means not normal or not homogenous
Step-down Jonckheere or Williams' test
Step-down Jonckheere test
Rep means normally distributed
Variances equal
Variances unequal
Dunnett test
variance stabilising transform?
Yes
No
<=3 reps per conc
>=4 reps per conc
Tamhane-Dunnett test
Dunn test
Dunn or Mann-Whitney test
Rep means not normally distributed
Yes
No
Nested ANOVA normal
Nested ANOVA not normal
Normalising transform?
Yes
No
<=3 reps per conc
>=4 reps per conc
Dunn test on rep means
Dunn or Mann-Whitney test on rep means
variance stabilising transform?
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No
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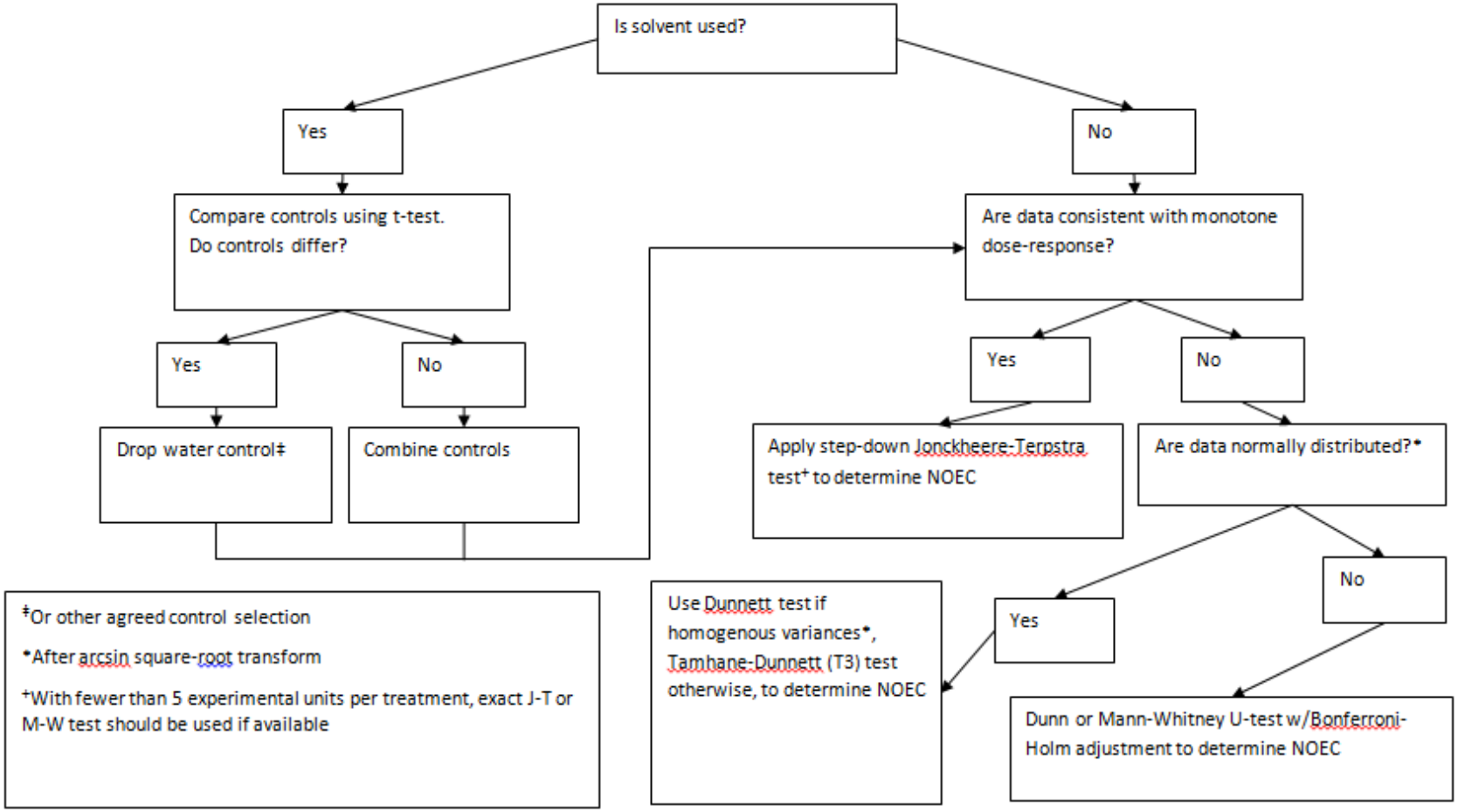
Tamhane-Dunnett test on nested ANOVA

STATISTICAL FLOW CHART FOR SEX RATIO ANALYSIS



APPENDIX 9

GUIDANCE FOR TISSUE SAMPLING FOR GENETIC SEX DETERMINATION AND FOR GENETIC SEX DETERMINATION BY PCR-METHOD

Tissue sampling, preparation and storage before determination of genetic sex by PCR-method in medaka (Prepared by the Laboratory for Aquatic Organisms of Bayer CropScience AG)

1. With fine scissors the anal or the dorsal fin will be cut off in each individual fish and placed into a tube filled with 100 µl of extraction-buffer 1 (details on buffer preparation see below). The scissors will be cleaned after each single fish in a beaker filled up with distilled H_2O and dried with a paper tissue.

2. Now the fin-tissues will be homogenised by a micro tube teflon pistil for the lysis of cells. For each tube a new pistil will be used to prevent any contaminations. The pistils will be placed overnight in 0.5 M NaOH, rinse for 5 minutes in distilled H_2O and stored in ethanol or sterile after autoclave until use.

3. It is also possible to store the fin tissue without any extraction-buffer 1 on dry-ice and then at -80°C refrigerator to prevent any degeneration of the DNA. But the extraction runs better, if you extract the DNA at the same time (handling see above; samples should be thawed on ice after storaging at -80°C before the buffer will be filled in the tubes).

4. After homogenizing all tubes will be placed in a water bath and boiled for 15 minutes at 100°C.

5. Then 100 µl of the extraction buffer 2 (details on buffer preparation see below) will be pipetted into each tube. The samples will be stored at room temperature for 15 minutes and in the meantime they will be sometimes gently shaken by hand.

6. Afterwards all tubes will be placed in the water bath again and boiled for another 15 minutes at 100°C.

7. Until further analysis the tubes will be frozen at -20°C.

Buffer preparation

PCR-buffer 1:

500 mg N-Lauroylsarcosine (e.g. Merck KGaA, Darmstadt, GE)

2 ml 5M NaCl

ad 100 ml dest. H_2O

→ autoclave

PCR-buffer 2:

20 g Chelex (e.g. Biorad, Munich, GE)

To swell in 100 ml dest. H_2O

→ autoclave

Determination of genetic sex (by PCR-method) in medaka (Prepared by the Laboratory for Aquatic Organisms of Bayer CropScience AG and Universität Würzburg Biozentrum)

The prepared and frozen tubes (described in the above section) will be thawed on ice. After that, they will be centrifuged using an Eppendorf centrifuge (30sec at max. speed, at room temperature). For the PCR, the clear supernatant separated from the precipitate will be used. It has absolutely to be avoided that any traces of Chelex (localized in the precipitate) are transferred to the PCR reaction, because this will interfere with the "Taq"-polymerase activity. The supernatant will be used directly or can be stored frozen (at -20 °C) and rethawed again in several cycles without negative impact on the DNA for later analyses.

1. *Preparation of the "Reaction Mix" (25 µl per sample):*

	Volume	Final Concentration
Template DNA	0.5µl-2µl	
10xPCR-buffer with MgCl2	2.5µl	1x
Nucleotides (each of dATP, dCTP, dGTP, dTTP)	4µl (5mM)	200µM
Forward Primer (10µM) (see below 3-5)	0.5µl	200nM
Reverse Primer (10µM) (see below 3-5)	0.5µl	200nM
DMSO	1.25µl	5%
Water (PCR grade)	up to 25µl	
Taq E- Polymerase	0.3µl	1.5U

10xPCR-buffer with $MgCl_2$: 670mM Tris/HCl (pH8.8 at 25°C), 160mM $(NH_4)_2SO_4$, 25mM MgCl2, 0.1%Tween 20

For each PCR (see below 3-5) the special primer as a new combination of "Reaction-Mix" and the adequate needed amount of template DNA for each sample (see above) is needed. The respective volumes will be transferred into new tubes using pipettes. After that all tubes will be closed, stirred (ca. 10 sec) and centrifuged (10 sec, at room temperature). Now the respective PCR-programmes can be started. Additionally a positive control (exemplary DNA sample with known activity and clear results) and a negative control (1 µl dest. H_2O) will be used in each PCR-programme.

2. Preparation of the agarose gel (1 %) – During running PCR-programmes:

- Solve 3 g agarose in 300ml 1 x TAE-buffer (1% agarose gel)
- This solution should be boiled using an microwave (ca. 2-3 min)
- Transfer the hot solution into a special casting box, which lies on ice
- After ca. 20 min the agarose gel is ready to use
- Storage the agarose gel in 1x TAE-buffer until the end of the PCR-programmes

3. Actin-PCR-programme:

This PCR-reaction is aimed to demonstrate that the DNA in the sample is not harmed.

- Special primer:

  "Mact1(upper/forward)" ➔ TTC AAC AGC CCT GCC ATG TA

  "Mact2(lower/reverse)" ➔ GCA GCT CAT AGC TCT TCT CCA GGG AG

- Programme:

  5 min 95 °C

  Cycle (35-times):

Denaturation	➔ 45 sec at 95 °C
Annealing	➔ 45 sec at 56 °C
Elongation	➔ 1 min at 68 °C

  15 min 68 °C

4. X- and Y-Gene-PCR-programme:

The samples with intact DNA will be used in this PCR-programme to detect the X- and Y-Genes. Male DNA should show one double-band and female DNA should show one single band (after staining and gel-electrophoresis). For this programme-run one positive control for males (XY-sample) and one for females (XX-sample) should be included.

- Special primer:

  "PG 17.5" (upper/forward) ➔ CCG GGT GCC CAA GTG CTC CCG CTG

  "PG 17.6" (lower/reverse) ➔ GAT CGT CCC TCC ACA GAG AAG AGA

- Programme:

  5 min 95 °C

  Cycle (40-times):
  Denaturation ➔ 45 sec at 95 °C
  Annealing ➔ 45 sec at 55 °C
  Elongation ➔ 1 min 30 sec at 68 °C

  15 min 68 °C

5. Y-Gene-PCR-programme as "control" for X- and Y-Gene-PCR-programme:

This PCR-programme verifies the results of the "X- and Y-Gene-PCR-programme". The "male-samples" should show one band and the "female-samples" shouldn't show any band (after staining and gel-electrophoresis).

- Special primer:

  "DMTYa (upper/forward)" ➔ GGC CGG GTC CCC GGG TG

  "DMTYd (lower/reverse)" ➔ TTT GGG TGA ACT CAC ATG G

- Programme:

  5 min 95 °C

  Cycle (40-times):
  Denaturation ➔ 45 sec at 95 °C
  Annealing ➔ 45 sec at 56 °C
  Elongation ➔ 1 min at 68 °C

  15 min 68 °C

6. Staining of the PCR-samples:

Staining solution:
50 % Glycerol
100 mM EDTA
1 % SDS
0.25 % Bromphenolblue
0.25 % Xylenecyanol

Pipette 1 µl of the staining solution into each single tube

7. Start of the Gel-Electrophoresis:

- The prepared 1 % agarose gel will be transferred into a gel-electrophoresis-chamber filled with 1 x TAE-Buffer
- 10 - 15 µl of each stained PCR-sample will be pipetted into an agarose gel slot
- Also 5 - 15 µl of the 1kb-“Ladder”(Invitrogen) will be pipetted into a separate slot
- Start the electrophoresis by 200 V
- Stop after 30-45 min

8. Determination of the bands:

- Clean the agarose gel in distilled H2O
- Now transfer the agarose gel into Ethidium bromide for 15 - 30 min
- After that, a picture of the agarose gel should be taken in an UV-light-box
- Finally the samples are analysed in comparison to the positive control-band (or bands) and the ladder

APPENDIX 10

GUIDANCE ON TISSUE SAMPLING FOR GENETIC SEX DETERMINATION BY PCR METHOD IN THE THREE-SPINED STICKLEBACK

Tissue sampling and DNA extraction

DNA can be extracted using a variety of commercially available reagents and both manual and automated extraction systems. The protocol used at the Cefas Weymouth laboratory is outlined below, and the alternative approaches have been added where appropriate.

1. With fine scissors, a small piece of tissue (10-20 mg) from the dorsolateral area (after removing the head and tail for VTG analysis), is removed from each individual fish. The tissue is added into a tube and either placed directly in liquid nitrogen (for storage at -80°C) or filled with 70% ethanol (for transport and subsequent storage at 4°C). The scissors are cleaned after each single fish in 70% ethanol then in distilled water and dried with tissue paper.

2. The ethanol (if present) is removed by aspiration and the tissue is digested overnight with proteinase K in 400 µl of ATL buffer (Qiagen). An aliquot (200 µl) of the digest is transferred to a 96-well S-block (Qiagen) and the DNA extracted in a 96-well format using the Qiagen Universal BioRobot and the QIamp Investigator BioRobot kit. The DNA is eluted in a 50 µl of DNase and RNase free water. If using hard tissues to extract DNA (such as a spine or a pectoral fin) it may be necessary to homogenise the sample in the lysis buffer using a FastPrep® tissue lyser or equivalent tissue disruption system.

Alternatively,

(a) the tissue is digested overnight with proteinase K in 400 µl of G2 lysis buffer (Qiagen) and DNA is extracted from 200 µl of the digest using either the EZ-1 DNA easy tissue kit and the EZ-1 biorobot or the DNA easy tissue mini kit. The DNA is eluted in a 50 µl volume.

(b) The tissues are processed using the DNAzol reagent. Briefly, tissue samples are lysed in 1ml of DNAzol for 10 minutes in a 1.5 ml micro centrifuge tube and then centrifuged at 13,000 rpm for 5 minutes to remove any particulate matter. The lysed sample is then transfered to a new 1.5 ml micro centrifuge tube containing 500 µl of 100% molecular grade ethanol and then centrifuged at 13,000 rpm for 10 minutes to precipitate the DNA. The ethanol is removed and replaced with 400 µl of 70% molecular grade ethanol, centrifuged at 13,000 rpm for 5 minutes and the DNA pellet is dissolved in 50 µl molecular DNase and RNase free water. Again, when using the hard tissues (pectoral fin) it may be necessary to homogenise the sample in the lysis buffer using a FastPrep® tissue lyser or equivalent tissue disruption system prior to extracting the DNA.

3. The DNA is stored at -20°C until required.

Important note: gloves must be worn during the procedures.

Polymerase chain reaction (PCR) analysis

Amplifications were performed using 2.5 µl of the DNA extract in a 50 µl reaction volume using the Idh locus primers (as described by Peichel et al., 2004. Current Biology 1:1416-1424):

Forward primer 5' GGG ACG AGC AAG ATT TAT TGG 3'

Reverse primer 5' TAT AGT TAG CCA GGA GAT GG 3'

There are numerous suppliers of suitable PCR reagents. The method outlined below is that currently used at the Cefas Weymouth laboratory.

1. Preparation of the "Reaction Mix" (50 µl per sample):

A mastermix is prepared as follows. This can be prepared in advance and stored frozen at -20 °C until required. Make sufficient mastermix for a negative control (molecular biology grade water only).

	Volume (stock conc.)/ sample	Final Concentration
5xGoTaq® Reaction Buffer	10µl	1x
$MgCl_2$	5 µl (25 mM)	2.5 mM
Nucleotides (dATP, dCTP, dGTP, dTTP)	0.5 µl (25 mM each)	250 µM each
Forward Primer	0.5µl (0.1 nmol/µl)	2.0 µM
Reverse Primer	0.5µl (0.1 nmol/µl)	2.0µM
Molecular biology grade water	30.75 µl	
GoTaq polymerase	0.25 µl	1.25U

- Dispense 47.5 µl to a labelled 0.5ml thin walled PCR tube.

- Add 2.5 µl of the purified DNA to the appropriately labelled tube. Repeat for all samples and the negative control.

- Over lay with 2 drops of mineral oil. Alternatively, use a thermal cycler with a heated lid.

- Close the lids.

- Samples were denatured in a Peltier PTC-225 thermal cycler at 94 ± 2°C for 5 minutes followed by 39 cycles of 94 ± 2°C for 1 minute, 55 ± 2°C for 1 minute, 72 ± 2°C for 1 minute, and a final extension of 72 ± 2°C for 10 minutes.

2. Preparation of the agarose gel (2 %):

Traditionally the PCR products are resolved on a 20% agarose gel containing ethidium bromide.

Capillary based electrophoresis systems can also be used.

- Weigh 2 g agarose in 100 ml 1 x TAE-buffer
- Heat in a microwave (ca. 2-3 min) to dissolve the agarose.
- Add 2 drops of ethidium bromide final concentration 0.5µg/ml
- Transfer the hot solution into the gel casting equipment.
- Allow the gel to harden

3. Gel-Electrophoresis:

- Transferred the agarose gel to the electrophoresis equipment and submerge in 1 x TAE-buffer
- Load 20 µl of each sample to a separate well, adding a molecular weight marker (100bp DNA ladder, Promega) to a spare well.
- Electrophoresis is performed at 120 V for 30-45 minutes.

4. Visualisation of the amplification products

If the ethidium bromide was incorporated in to the agarose gel as described above, the DNA products are visualised under a UV source. Alternatively the agarose gel is stained by covering the gel in a dilute solution of ethidium bromide (0.5 µg/ml in water) for 30 minutes prior to visualisation.

APPENDIX 11

GUIDANCE FOR ARTIFICIAL FERTILISATION PROCEDURE FOR THE THREE-SPINED STICKLEBACK

The purpose of this section is to describe the procedures to obtain fertilised eggs from the three-spined stickleback in view of using them in the FSDT.

Procedures

Obtaining sperm from the males

1. A well-coloured male of the desired population is euthanised.

2. The testes are dissected from each side of the fish. *The testes are generally heavily pigmented, rod shaped structures that are readily apparent at the lateral midline of the body*. Use either of the following methods:

3. Using a pair of fine scissors, begin at the cloaca and make a 1-1.5cm incision with a single snip angled at about 45 degrees.

4. Use a scalpel to make a small incision in the side of the fish slightly posterior to the pelvis and just ventral of the lateral plates.

5. The testes are removed using fine forceps and placed into a petri dish.

6. Each testis is covered with 100 µl freshly made **Hank's final solution***.

7. The testes are finely diced by using a razor blade or scalpel. This will release sperm and give the Hank's solution a milky appearance.

8. The fluid containing sperm is added into a tube, while trying not to include any pieces of testes tissue when pipetting.

9. 800 µl of Hank's final solution are added into the tube and mixed well.

10. If required, the male can be preserved by fixing in 100% ethanol or other desired fixative. This is particularly important if the study is assigning parental origin of offsprings.

* Hank's Buffered Salt Solution (HBSS):

HBSS is needed to preserve the sperm whilst preparing for fertilisation.

Important note: *Although most of the stock solutions required can be made in advance**, stock 5** and subsequently the **final solution**, should be made up **fresh** on the day of use.*

Stock 1

NaCl	8.00 g

KCl	0.40 g
Distilled water (DW)	100 ml

Stock 2

Na_2HPO_4 (anhydrous)	0.358 g
KH_2PO_4	0.60 g
DW	100 ml

Stock 3

$CaCl_2$	0.72 g
DW	50 ml

Stock 4

$MgSO_4 \cdot 7H_2O$	1.23 g
DW	50 ml

Stock 5 (freshly prepared)

$NaHCO_3$	0.35 g
DW	10 ml

Note: If you already have some of the above salts but with different water content (i.e. $2H_2O$ instead of anhydrous) you can still use it but first adjust weight based on molecular weight).

For Hank's final solution combine in the following order:

stock 1	1.0 ml
stock 2	0.1 ml
stock 3	0.1 ml
DW	8.6 ml
stock 4	0.1 ml
stock 5	0.1 ml

Mix well before use.

Fertilisation

1. Large, gravid females are identified from the desired population; females are ready for squeezing only when you can see eggs protruding from the cloaca. Ready females have the characteristic 'head up' posture.

2. Gently run a finger or thumb down the side of the fish towards the tail to encourage the expulsion of an egg sack into a fresh petri dish. Repeat on the other side and return the fish to its tank.

3. The eggs can be spread out (forming a monolayer) using a fine paintbrush. It is important to try and expose as many eggs as possible to the sperm so maximising the surface area of the eggs is helpful. Important note: Keep the

eggs humid by laying damp tissue around them (it is important the eggs do not touch water directly as this can prematurely harden the chorion preventing fertilisation). There is a large variation in the number of eggs each female can produce but as an average, about 150 eggs should be easily obtained from a single gravid female.

4. 25µl of sperm in Hank's mixture is spread evenly over the whole surface of the eggs using the paintbrush. The eggs will quickly harden and change colour (within a minute) once fertilisation has begun. If the estimated number of eggs is more than 150, repeat the procedure. Similarly if the eggs don't harden within a minute add a bit more sperm. Important note: Adding more sperm does not necessarily improve fertilisation rate.

5. The eggs and the sperm solution should be left to 'interact' for at least 15 minutes and the fertilised eggs should be placed into the exposure aquaria within 1.5 hours post fertilisation.

6. The procedure is repeated using another female until the desired number of eggs is collected.

7. Spare few eggs from the last batch and fix them in 10% acetic acid.

Counting and distributing eggs in test aquaria

1. Eggs should be evenly distributed between each treatment level to avoid genetic bias. Each batch of fertilised eggs should be separated into equal size groups (as many as the treatment levels) by the use of a blunt instrument (i.e. wide-blade entomology forceps or use of an inoculation loop). If you aim for 4 replicates per treatment, with 20 eggs each then you need to distribute 80 eggs per exposure aquaria. Important note: It is advisable to add an extra 20% (i.e. 96 eggs per treatment level) until you are confident that you obtain 100% fertilisation rates.

2. Stickleback eggs are very prone to fungal infections outside the father-guarded nest. In this respect, treatment of all eggs with methylene blue during the first 5 days of the test is critically important. A stock solution of methylene blue is prepared at 1 mg/ml and added to the exposure aquaria to give a maximum final concentration of 2.125 mg/l. Important note: Sticklebacks should not be exposed to methylene blue once hatched so the system should be free of methylene blue by day 6.

3. The eggs are inspected daily and any dead or unfertilised eggs are recorded as such. Important note: The eggs should never be outside water until they hatch even for very brief periods.

C.42 Biodegradability in Seawater

GENERAL INTRODUCTION

1. This test method is equivalent to OECD Test Guideline (TG) 306 (1992). When the original test methods were developed, it was not known to what extent results from the screening tests for ready biodegradability using freshwater, and sewage effluent or activated sludge as inoculum, could be applied to the marine environment. Variable results on this point have been reported (e.g. (1)).

2. Many industrial waste waters, containing a variety of chemicals, reach the sea either by direct discharge or via estuaries and rivers in which the residence times are low compared with the period necessary for complete biodegradation of many of the chemicals present. Because of the growing awareness of the need to protect the marine environment against increasing loads of chemicals and the need to estimate the probable concentration of chemicals in the sea, test methods for biodegradability in seawater have been developed.

3. The methods described here use natural seawater both as the aqueous phase and as the source of micro-organisms. In an endeavour to conform with the methods for ready biodegradability in freshwater, the use of ultra-filtered and centrifuged seawater was investigated, as was the use of marine sediments as inocula. These investigations were unsuccessful. The test medium therefore is natural seawater pre-treated to remove coarse particles.

4. In order to assess ultimate biodegradability with the Shake Flask Method, relatively high concentrations of the test substance have to be used because of the poor sensitivity of the dissolved organic carbon (DOC) analytical method. This in turn necessitates the addition to the seawater of mineral nutrients (N and P), the low concentrations of which would otherwise limit the removal of DOC. It is also necessary to add the nutrients in the Closed Bottle Method because of the concentration of the added test substance.

5. Hence, the methods are not tests for ready biodegradability since no inoculum is added in addition to the micro-organisms already present in the seawater. Neither do the tests simulate the marine environment since nutrients are added and the concentration of test substance is very much higher than would be present in the sea. For these reasons the methods are proposed under a new subsection "Biodegradability in Seawater".

APPLICATION

6. The results of the tests, which would be applied because the pattern of use and disposal of the substance in question indicated a route to the sea, give a first impression of biodegradability in seawater. If the result is positive (>70% DOC removal; >60%

ThOD - theoretical oxygen demand), it may be concluded that there is a potential for biodegradation in the marine environment. However, a negative result does not preclude such a potential but indicates that further study is necessary, for example, using as low a concentration of the test substance as possible.

7. In either case, if a more definitive value for the rate or degree of biodegradation in seawater at a particular site is required, other more complex and sophisticated, and hence more costly, methods would have to be applied. For example, a simulation test could be applied using a concentration of test substance nearer to the likely environmental concentration. Also, non-fortified, non-pre-treated seawater taken from the location of interest could be used and primary biodegradation could be followed by specific chemical analysis. For ultimate biodegradability, ^{14}C-labelled substances would be necessary in order that the rates of the disappearance of soluble organic ^{14}C and the production of $^{14}CO_2$ at environmentally realistic concentrations could be measured.

CHOICE OF METHODS

8. The selection of which method to use depends on a number of factors; the following Table is given to help the selection. While substances of water solubility below the equivalent of about 5 mg C/l cannot be tested in the Shake Flask Method, at least, in principle, poorly soluble substances may be tested in the Closed Bottle Method.

Table: Advantages and disadvantages of the shake flask and closed bottle test

METHOD	ADVANTAGES	DISADVANTAGES
SHAKE FLASK	- simple apparatus except C analyser - 60 d duration is not a problem - no interference from nitrification - can be adapted for volatile substances	- needs C analyser - uses 5-40 mg DOC/1, could be inhibitory - DOC determination is difficult at low concentrations in seawater (chloride effect) - DOC sometimes high in seawater
CLOSED BOTTLE	- simple apparatus - simple end determination - uses low concentration of test substance (2 mg/l) thus less chance of inhibition - easily adapted for volatile substances	- could be difficult to maintain air-tightness of bottles - wall growth of bacteria can lead to false values - blank O_2 uptake values can be high especially after 28 days; could be overcome by ageing the seawater - possible interference from O_2 uptake by nitrification

SHAKE FLASK METHOD

INTRODUCTION

1. This method is a seawater variant of the Modified OECD Screening Test described in Chapter C.4B of this Annex (2). It was finalised as a result of a ring test organized for the European Commission (EC) by the Danish Water Quality Institute (3).

2. In common with the accompanying marine Closed Bottle Method, the results from this test are not to be taken as indicators of ready biodegradability, but are to be used specifically for obtaining information about the biodegadability of substances in marine environments.

PRINCIPLE OF THE METHOD

3. A pre-determined amount of the test substance is dissolved in the test medium to yield a concentration of 5-40 mg dissolved organic carbon (DOC)/l. If the limits of sensitivity of organic carbon analyses are improved, the use of lower concentrations of test substance may be advantageous, particularly for inhibitory substances. The solution of the test substance in the test medium is incubated under agitation in the dark or in diffuse light under aerobic conditions at a fixed temperature (controlled to ± 2°C) which will normally be within the range 15-20°C. In cases where the objective of the study is to simulate environmental situations, tests may be carried out beyond this normal temperature range. The recommended maximum test duration is about 60 days. Degradation is followed by DOC measurements (ultimate degradation) and, in some cases, by specific analysis (primary degradation).

INFORMATION ON THE TEST SUBSTANCE

4. In order to know whether the test may be applied to a particular substance, some of its properties must be known. The organic carbon content of the substance must be established, its volatility must be such that significant losses do not occur during the course of the test and its solubility in water should be greater than the equivalent of 25-40 mg C/l. Also, the test substance should not significantly adsorb onto glass surfaces. Information on the purity or the relative proportions of major components of the test substance is required in order that the results obtained can be interpreted, especially when the result lies close to the "pass" level.

5. Information on the toxicity of the test substance to bacteria, for example as measured in short-term respiration rate tests (4), may be useful when selecting appropriate test concentrations and may be essential for the correct interpretation of low biodegradation values. However, such information is not always sufficient for interpreting results obtained in the biodegradation test and the procedure described in paragraph 18 is more suitable.

REFERENCE SUBSTANCES

6. Suitable reference substances must be used to check the microbial activity of the seawater sample. Sodium benzoate, sodium acetate and aniline are examples of substances which may be used for this purpose. The reference substances must be degraded within a reasonably short time span, otherwise it is recommended that the test be repeated using another seawater sample.

7. In the EC ring test where seawater samples were taken at different locations and at different times of the year (3), the lag phase (t_L) and time to achieve 50 per cent degradation (t_{50}), excluding the lag phase, were 1 to 4 days and 1 to 7 days respectively for sodium benzoate. For aniline the t_L ranged from 0 to 10 days, whilst the t_{50} ranged from 1 to 10 days.

REPRODUCIBILITY AND SENSITIVITY OF THE METHOD

8. The reproducibility of the method was established in the ring test (3). The lowest concentration of test substance, for which this method can be used with DOC analysis, is largely determined by the detection limit of the organic carbon analysis (about 0.5 mg C/l, at present) and the concentration of dissolved organic carbon in the seawater used (usually of the order of 3-5 mg/l for water from the open sea). The background concentration of DOC should not exceed about 20% of the total DOC concentration after addition of test substance. If this is not feasible, the background concentration of DOC may sometimes be reduced by ageing the seawater prior to testing. If the method is used with specific chemical analysis only (by which primary degradation is measured), the investigator must document, by supplying additional information, whether ultimate degradability can be expected. This additional information may consist of the results from other tests for ready or inherent biodegradability.

DESCRIPTION OF THE METHOD

Apparatus

9. Normal laboratory apparatus and:

   a. Shaking machine accommodating 0.5-2 litre Erlenmeyer flasks, either with automatic temperature control or used in a constant temperature room at 15-20°C controlled to ± 2°C;

   b. Narrow neck, 0.5-2 litre Erlenmeyer flasks;

   c. Membrane filtration apparatus, or centrifuge;

   d. Membrane filters, 0.2-0.45μm;

   e. Carbon analyser;

   f. Equipment for specific analysis (optional).

Seawater

10. Collect a sample of seawater in a thoroughly cleansed container and transport to the laboratory, preferably within one or two days of collection. During transport, do not allow the temperature of the sample to exceed significantly the temperature to be used in the test. Identify the sampling location precisely and describe it in terms of its pollutional and nutrient status. Especially for coastal waters, include in this characterization a heterotrophic microbial colony count and the determination of the concentrations of dissolved nitrate, ammonium and phosphate.

11. Provide the following information for the seawater sample itself:

- date of collection;
- depth of collection;
- appearance of sample - turbid, etc.;
- temperature at the time of collection;
- salinity;
- DOC;
- delay between collection and use in the test.

12. If the DOC content of the seawater sample is found to be high (paragraph 8), it is recommended that the seawater be aged for about a week prior to use. Age by storing under aerobic conditions at the test temperature and in the dark or in diffuse light. If necessary, maintain aerobic conditions by gentle aeration. During ageing, the content of easily degradable organic material is reduced. In the ring test (3), no difference was revealed between the degradation potential of aged and freshly collected seawater samples. Prior to use, pre-treat the seawater to remove coarse particles, e.g. by filtration through a nylon filter or coarse paper filter (not membrane or GF-C filters), or by sedimentation and decanting. The procedure used must be reported. Carry out pre-treatment after ageing, if used.

Stock solutions for mineral nutrients

13. Prepare the following stock solutions, using analytical grade reagents:

(a)	Potassium dihydrogen orthophosphate, KH_2PO_4	8.50 g
	Dipotassium hydrogen orthophosphate, K_2HPO4	21.75 g
	Disodium hydrogen orthophosphate dihydrate, $Na_2HPO_4 \cdot 2H_2O$	33.30 g
	Ammonium chloride, NH_4Cl	0.50 g
	Dissolve and make up to 1 litre with distilled water.	
(b)	Calcium chloride, $CaCl_2$	27.50 g
	Dissolve and make up to 1 litre with distilled water.	
(c)	Magnesium sulphate heptahydrate, $MgSO_4 \cdot 7H_2O$	22.50 g
	Dissolve and make up to 1 litre with distilled water.	
(d)	Iron (III) chloride hexahydrate, $FeCl_3 \cdot 6H_2O$	0.25 g
	Dissolve and make up to 1 litre with distilled water.	

Precipitation in solution (d) may be prevented by adding one drop of concentrated HCl or 0.4 g ethylenediaminetetra-acetic acid (EDTA, disodium salt) per litre. If a precipitate forms in a stock solution, replace it with freshly made solution.

Preparation of test medium

14. Add 1 ml of each of the above stock solutions per litre of pre-treated seawater.

Inoculum

15. Do not add a specific inoculum in addition to the micro-organisms already present in the seawater. Determine (optionally) the number of colony-forming heterotrophs in the seawater test medium (and preferably also in the original seawater samples) e.g. by plate count, using marine agar. This is particularly desirable for samples from coastal or polluted sites. Check the heterotrophic microbial activity in the seawater by performing a test with a reference substance.

Preparation of flasks

16. Ensure that all glassware is scrupulously clean, not necessarily sterile, (e.g. using alcoholic hydrochloric acid), rinsed and dried before use in order to avoid contamination with residues from previous tests. The flasks must also be cleaned before first use.

17. Evaluate test substances in duplicate flasks simultaneously, together with a single flask for the reference substance. Carry out a blank test, in duplicate, with neither test nor

reference substance for the determination of analytical blanks. Dissolve the test substances in the test medium - they may be conveniently added via a concentrated stock solution - to give the desired starting concentrations of normally 5-40 mg DOC/l. Test the reference substance normally at a starting concentration corresponding to 20 mg DOC/l. If stock solutions of test and/or reference substances are used, ensure that the salinity of the seawater medium is not greatly altered.

18. If toxic effects can be expected or cannot be ruled out, it may be advisable to include an inhibition experiment, in duplicate, in the test design. Add the test and reference substances to the same vessel, the concentration of the reference substance being normally the same as in the control test (i.e. 20 mg DOC/l) in order to allow comparison.

19. Dispense adequate amounts of test solutions into the Erlenmeyer flasks (up to about half the flask volume is a convenient amount) and subsequently provide each flask with a loose cover (e.g. aluminium foil) that makes gas exchange between the flask and the surrounding air possible. (Cotton wool plugs are unsuitable if DOC analysis is used). Place the vessels on the shaker and shake continuously at a gentle rate (e.g. 100 rpm) throughout the test. Control the temperature (15-20°C and within ± 2°C), and shield the vessels from light in order to avoid growth of algae. Ensure that the air is free of toxic materials.

Physical-chemical control test (optional)

20. If abiotic degradation or loss mechanisms are suspected, such as hydrolysis (a problem with specific analysis only), volatilization, or adsorption, it is advisable to perform a physical-chemical control experiment. This can be done by adding mercury (II) chloride ($HgCl_2$)[(1)] (50-100 mg/l) to vessels with test substance in order to stop microbial activity. A significant decrease in DOC or specific substance concentration in the physical-chemical control test indicates abiotic removal mechanisms. (If mercury chloride is used, attention should be paid to interferences or catalyst poisoning in DOC analysis.)

Number of flasks

21. In a typical run, the following flasks are used:

Flasks 1 & 2 - containing test substance (test suspension);

Flasks 3 & 4 - containing seawater only (blank);

Flask 5 - containing reference substance (procedure control);

Flask 6 - containing test and reference subtance (toxicity control) - optional;

[(1)] Mercury (II) chloride ($HgCl_2$) is a very toxic substance which should be handled with suitable precautions. Aqueous wastes containing this chemical should be disposed of appropriately; they should not be discharged into the waste water system.

Flask 7 - containing test substance and sterilising agent (abiotic sterile control)-optional.

DOC analysis

22. In the course of the test, withdraw samples at suitable intervals for DOC analysis (Appendix 1). Always take samples at the start of the test (day 0) and at day 60. A minimum of five samples in total are required to describe the time-course of degradation. No fixed time schedule for sampling can be stated as the rate of biodegradation varies. Carry out the DOC determination in duplicate on each sample.

Sampling

23. The required volume of the samples depends upon the analytical method (specific analysis), on the carbon analyser used, and on the procedure (membrane filtration or centrifugation) selected for sample treatment before carbon determination (paragraphs 25 and 26). Before sampling ensure that the test medium is mixed well and that any material adhering to the wall of the flask is dissolved or suspended.

24. Membrane-filter or centrifuge immediately after sampling. If necessary, store the filtered or centrifuged samples at 2-4°C for up to 48 hours or below -18°C for longer periods (if it is known that the substance will remain unaffected, acidify to pH 2 before storing).

25. Membrane filters (0.2-0.45 μm) are suitable if it is ensured that they neither release carbon nor adsorb the substance in the filtration step e.g. polycarbonate membrane filters. Some membrane filters are impregnated with surfactants for hydrophilization and may release considerable quantities of dissolved carbon. Prepare such filters by boiling in deionised water for three consecutive periods, each of one hour. After boiling, store the filters in deionised water. Discard the first 20 ml of the filtrate.

26. Centrifugation of the samples may be chosen as an alternative to membrane filtration. Centrifuge at 40 000 $m.s^{-2}$ (~ 4000 g) for 15 minutes, preferably in a refrigerated centrifuge.

Note: The differentiation of Total Organic Carbon (TOC) over DOC (TOC/DOC) by centrifugation at very low concentrations does not seem to work, since either not all bacteria are removed, or carbon as part of the bacterial plasma is redissolved. At higher test concentrations (> 10 mg C per litre), the centrifugation error seems to be comparatively small.

Frequency of sampling

27. If analyses are performed immediately after sampling, assess the next sampling time by considering the result of the analytical determination.

28. If samples are preserved (paragraph 24) for analysis at a later time, take more samples than the required minimum number of five. Analyse the last samples first, and by a step-wise "backwards" selection of appropriate samples for analysis, it is possible to

obtain a good description of the biodegradation curve with a relatively small number of analytical determinations. If no degradation has taken place by the end of the test, no further samples need to be analysed, and in this situation, the "backwards" stategy may save considerable analytical costs.

29. If a plateau on the degradation curve is observed before the 60th day, end the test. If degradation has obviously started by day 60, but has not reached a plateau, extend the experiment for a further period.

DATA AND REPORTING

Treatment of results

30. Record the analytical results on the attached data sheet (Appendix 2), and calculate the biodegradation values for both test and reference substances from the equation:

$$D_t = \left[1 - \frac{C_t - C_{bl(t)}}{C_0 - C_{bl(0)}}\right] \times 100$$

where:
D_t = degradation in percentage DOC or specific substance removal at time t,
C_o = starting concentration of DOC or specific substance in the test medium,
C_t = concentration of DOC or specific substance in the test medium at time t,
$C_{bl(0)}$ = starting concentration of DOC or specific substance in the blank,
$C_{bl(t)}$ = concentration of DOC or specific substance in the blank at time t.

31. State degradation as the percentage DOC removal (ultimate degradation) or specific substance removal (primary degradation) at time t. Calculate the DOC concentrations to the nearest 0.1 mg per litre, and round up the means of the D_t values to the nearest whole per cent.

32. Illustrate the course of the degradation graphically in a diagram as shown in the figure in "Validity and interpretation of results". If there are sufficient data, calculate from the curve the lag phase (t_L) and the time to reach 50 per cent removal from the end of the lag phase (t_{50}).

Test report

33. The test report must contain the following information:

Test substance:
- physical nature and, where relevant, physicochemical properties;
- identification data.

Test conditions:
- location and description of the sampling site; pollutional and nutrient status (colony count, nitrate, ammonium, phosphate if appropriate);
- characteristics of the sample (date of sampling, depth, appearance, temperature,

salinity, DOC (optional), delay between collection and use in the test;
- method used (if any) for ageing of the seawater;
- method used for pre-treatment (filtration/sedimentation) of the seawater;
- method used for DOC determination;
- method used for specific analysis (optional);
- method used for determining the number of heterotrophs in the seawater (plate count method or alternative procedure) (optional);
- other methods (optional) used to characterise the seawater (ATP measurements, etc.).

Results:
- analytical data reported on a data sheet (Appendix 2);
- the course of the degradation test is represented graphically in a diagram showing the lag phase (t_L), slope, and time (starting from the end of the lag phase) to reach 50 per cent removal (t_{50}). The lag phase may be estimated graphically as shown in the figure in the "Validity and interpretation of results" section or conveniently taken as the time needed for 10 per cent degradation;
- percentage degradation measured after 60 days, or at end of test.

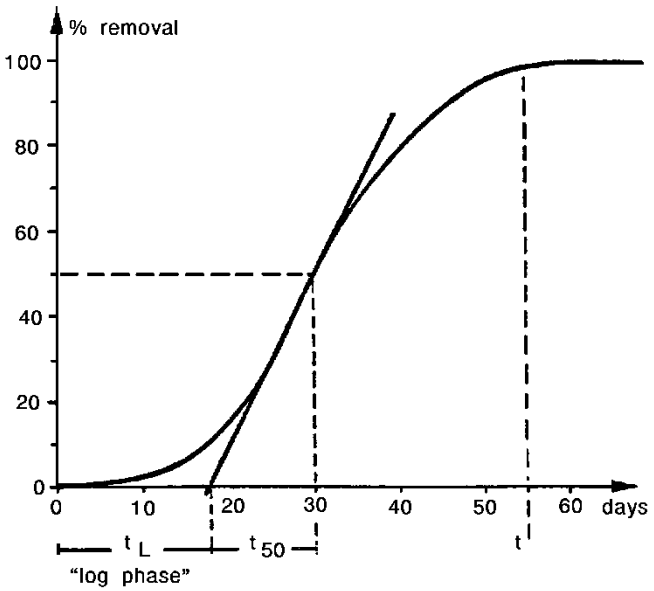
Discussion of results.

Validity and interpretation of results

34. The results obtained with the reference substances e.g. sodium benzoate, sodium acetate or aniline, should be comparable to results obtained in the ring test (3) (refer to section on "Reference substances", paragraph 7). If results obtained with reference substances are atypical, the test should be repeated using another seawater sample. Although results of inhibition tests may not always be straightforward to interpret because of the contribution of DOC by the test substance, a significant reduction of the total DOC removal rate, compared with that of the control, is a positive sign of toxic effects.

35. Owing to the relatively high test concentrations used as compared with most natural systems (and consequently an unfavourable ratio between the concentrations of test substances and other carbon sources), the method is to be regarded as a preliminary test which can be used to indicate whether or not a substance is easily biodegradable. Accordingly a low result does not necessarily mean that the test substance is not biodegradable in marine environments, but indicates that more work will be necessary in order for this to be established.

An example of a theoretical degradation experiment illustrating a feasible way of estimating the values of t_L (length of "lag phase") and t_{50} (time interval, starting at t_L), needed to reach 50 per cent removal, is given in the figure below.


% removal
100
80
60
40
20
0
0
10
20
30
40
50
60
days
t_L
t_{50}
t
"log phase"

CLOSED BOTTLE METHOD

INTRODUCTION

1. This method is a seawater variant of the Closed Bottle Test (5) and was finalised as a result of a ring test organised for the European Commission (EC) by the Danish Water Quality Institute (3).

2. In common with the accompanying marine Shake Flask Method, results of this test are not to be taken as indications of ready biodegradability, but are to be used specifically for obtaining information about the biodegradability of substances in marine environments.

PRINCIPLE OF THE METHOD

3. A pre-determined amount of the test substance is dissolved in the test medium in a concentration of usually 2-10 mg of test substance per litre (one or more concentrations may be used). The solution is kept in a filled closed bottle in the dark in a constant temperature bath or enclosure controlled to ± 1°C within a range of 15-20°C. In those cases where the objective of the study is to simulate environmental situations, tests may be carried out beyond this normal temperature range providing suitable adjustments are made for temperature control. The degradation is followed by oxygen analyses over a 28-day period.

4. The ring test showed that if the test was extended beyond 28 days no useful information could be gathered, in most cases, due to severe interferences. The blank biological oxygen demand (BOD) values were excessively high probably due to wall growth, caused by lack of agitation, and to nitrification. Thus, the recommended duration is 28 days, but if the blank BOD value remains within the 30 per cent limit (paragraphs 15 and 40) the test could be prolonged.

INFORMATION ON THE TEST SUBSTANCE

5. In order to know whether the test may be applied to a particular substance, some of its properties must be known. The empirical formula is required so that the theoretical oxygen demand (ThOD) may be calculated (see Appendix 3); otherwise the chemical oxygen demand (COD) of the substance must be determined to serve as the reference value. The use of COD is less satisfactory since some substances are not fully oxidised in the COD test.

6. The solubility of the substance should be at least 2 mg/l, though in principle less soluble substances could be tested (e.g. using ultra sonication) as could volatile substances. Information on the purity or the relative proportions of major components of the test substance is required in order that the results obtained can be interpreted, especially

when the result lies close to the "pass" level.

7. Information on the toxicity of the substance to bacteria e.g. as measured in short-term respiration tests (4) may be very useful when selecting appropriate test concentrations and may be essential for the correct interpretation of low biodegradation values. However, such information is not always sufficient for interpreting results obtained in the biodegradation test and the procedure described in paragraph 27 is more suitable.

REFERENCE SUBSTANCES

8. Suitable reference substances must be used to check the microbial activity of the seawater sample. Aniline, sodium acetate or sodium benzoate (for example) may be used for this purpose. A degradation of these substances of at least 60 per cent (of their ThOD) must occur within a reasonably short time span, otherwise it is recommended that the test be repeated using another seawater sample.

9. In the EC ring-test where seawater samples were taken at different locations and at different times of the year, the lag phase (t_L) and the time to achieve 50 per cent degradation (t_{50}), not including the lag phase, were 0 to 2 days and 1 to 4 days respectively for sodium benzoate. For aniline the t_L and t_{50} values were 0 to 7 and 2 to 12 days respectively.

REPRODUCIBILITY

10. The reproducibility of the methods was established in the EC ring test (3).

DESCRIPTION OF THE METHOD

Apparatus

11. Normal laboratory equipment and:

(a) 250-300 ml BOD bottles with glass stoppers or narrow neck 250 ml bottle with glass stoppers may be used;

(b) Several 2-, 3- and 4- litre bottles with litre marks for the preparation of the experiment and for the filling of the BOD bottles;

(c) Waterbath or constant temperature room for keeping the bottles at constant temperature (± 1°C) with the exclusion of light.

(d) Equipment for analysis of dissolved oxygen;

(e) Membrane filters, 0.2-0.45 μm (optional);

(f) Equipment for specific analysis (optional).

Seawater

12. Collect a seawater sample in a thoroughly cleansed container and transport to the laboratory, preferably within one or two days of collection. During transport do not allow the temperature of the sample to exceed significantly the temperature to be used in the test.

13. Identify the sampling location precisely and describe it in terms of its pollutional and nutritional status. Especially for coastal or polluted waters, include in this characterisation a heterotrophic microbial colony count and the determination of concentrations of dissolved nitrate, ammonium and phosphate.

14. Provide the following information for the seawater sample itself:

- date of collection;
- depth of collection;
- appearance of sample - turbid etc.;
- temperature at the time of collection;
- salinity;
- dissolved organic carbon (DOC);
- delay between collection and use in the test.

15. If the DOC content of the sample is found to be high or if it is thought that the blank BOD after 28 days would be more than 30 per cent of that of the reference substances, it is recommended that the seawater be aged for about a week prior to use.

16. Age the sample by storing it under aerobic conditions at the test temperature and in the dark or in diffuse light. If necessary, maintain aerobic conditions by gentle aeration. During ageing, the content of easily degradable organic material is reduced. In the ring-test (3), no difference was revealed between the degradation potential of aged and freshly collected seawater samples.

17. Prior to use, pretreat the seawater to remove coarse particles e.g. by filtration through a nylon filter or a coarse paper filter (not membrane or GF-C filters), or by sedimentation and decanting. Report the procedure used. Pretreat after ageing, if used.

Stock solutions for mineral nutrients

18. Prepare the following stock solutions using analytical grade reagents:

(a) Potassium dihydrogen orthophosphate, KH_2PO_4 8.50 g

	Dipotassium hydrogen orthophosphate, K_2HPO_4	21.75 g
	Disodium hydrogen orthophosphate dihydrate, $Na_2HPO_4 \cdot 2H_2O$	33.30 g
	Ammonium chloride, NH_4Cl	0.50 g
	Dissolve and make up to 1 litre with distilled water.	
(b)	Calcium chloride, $CaCl_2$	27.50 g
	Dissolve and make up to 1 litre with distilled water.	
(c)	Magnesium sulphate heptahydrate, $MgSO_4 \cdot 7H_2O$	22.50 g
	Dissolve and make up to 1 litre with distilled water.	
(d)	Iron (III) chloride hexahydrate, $FeCl_3 \cdot 6H_2O$	0.25 g
	Dissolve and make up to 1 litre with distilled water.	

Precipitation in solution (d) may be prevented by adding one drop of concentrated HCl or 0.4 g ethylenediaminetetra-acetic acid (EDTA, disodium salt) per litre. If a precipitate forms in a stock solution, replace it with freshly made solution.

Preparation of test medium

19. Add per litre of pre-treated seawater 1 ml of each of the above stock solutions. Saturate the test medium with air at the test temperature by aerating with clean compressed air for about 20 minutes. Determine the concentration of dissolved oxygen for control purposes. The saturated concentration of dissolved oxygen as a function of salinity and temperature may be read from the nomogram enclosed with this test method (Appendix 4).

Inoculum

20. Do not add a specific inoculum in addition to the micro-organisms already present in the seawater. Determine (optionally) the number of colony-forming heterotrophs in the seawater test medium (and preferably also in the original seawater sample), e.g. by plate count using a marine agar. This is particularly desirable for samples from coastal or polluted sites. Check the heterotrophic microbial activity in the seawater by performing a test with a reference substance.

Preparation of test bottles

21. Perform all necessary manipulations including ageing and pre-treatment of the seawater at the chosen test temperature between 15 to 20°C, ensuring cleanliness, but not sterility of all glassware.

22. Prepare groups of BOD bottles for the determination of the BOD of the test and reference substances in simultaneous experimental series. Perform all analyses on duplicate bottles (blanks, reference and test substances), i.e. prepare two bottles for each determination. Perform analyses at least on days 0, 5, 15 and 28 (four determinations). For oxygen analyses, four determinations require a total of 3 x 2 x 4 = 24 bottles (blank, reference and test substance), and thus about 8 litres of test medium (for one concentration of test substance).

23. Prepare separate solutions of test and reference substances in large bottles of sufficient volume (paragraph 11) by first adding test and reference substances either directly or by using a concentrated stock solution to the partly filled large bottles. Add further test medium to give the final desired concentrations. If stock solutions of test and/or reference substances are used, ensure that the salinity of the seawater medium is not significantly altered.

24. Select concentrations of test and reference substances by taking into account:

    (a) the solubility of dissolved oxygen in seawater at the prevailing test temperature and salinity (see the enclosed nomogram - Appendix 4);

    (b) the blank BOD of the seawater; and

    (c) the expected biodegradability of the test substance.

25. At 15°C and 20°C and 32 parts per thousand salinity (ocean water), the solubility of dissolved oxygen is about 8.1 and 7.4 mg/l respectively. The oxygen consumption of the seawater itself (blank respiration) may be 2 mg O_2/l or more, if the seawater is not aged. Therefore in order to ensure a significant oxygen concentration remaining after oxidation of the test substance, use a starting concentration of test substance of about 2-3 mg/l (depending on the ThOD) for the substances that are expected to become

completely degraded under the conditions of the test (such as reference substances). Test less degradable substances at higher concentrations, up to about 10 mg/l, provided that toxic effects do not occur. It can be advantageous to run parallel tests with a low (about 2 mg/l) and a high (about 10 mg/l) concentration of test substance.

26. An oxygen blank must be determined in parallel in bottles containing neither test or reference substance.

27. If inhibitory effects are to be determined, prepare the following series of solutions in separate large bottles (paragraph 13):

    (a) 2 mg per litre of an easily-degradable substance, e.g. any of the reference substances mentioned;

    (b) x mg per litre of test substance (x is usually 2);

    (c) 2 mg per litre of the easily-degradable substance plus x mg per litre of test substance.

Physical-chemical control test (optional)

28. If the option of using specific analyses is used, a physical-chemical experiment may be performed in order to check whether the test substance is removed by abiotic mechanisms, such as hydrolysis or adsorption. A physical-chemical control test may be performed by adding mercury (II) chloride ($HgCl_2$)[(1)] (50-100 mg/l) to duplicate flasks with test substance in order to stop microbial activity. A significant decrease in specific substance concentration in the course of the test indicates abiotic removal mechanisms.

Number of BOD bottles in a typical run

29. In a typical run the following bottles are used:

- at least 8 containing test substance;
- at least 8 containing nutrient-fortified seawater only;
- at least 8 containing reference substance, and when necessary
- 6 bottles containing test and reference substances (toxicity control).

PROCEDURE

30. After preparation, immediately siphon each solution, from the lower quarter (not from the bottom) of the appropriate large bottle, to fill the respective group of BOD bottles.

[(1)]Mercury (II) chloride ($HgCl_2$) is a very toxic substance which should be handled with suitable precautions. Aqueous wastes containing this chemical should be disposed of appropriately; they should not be discharged directly into the waste water system.

Immediately analyse the zero controls (time zero) for dissolved oxygen (paragraph 33) or preserve them for later chemical analysis by precipitation with $MnCl_2$ (manganese (II) chloride) and NaOH (sodium hydroxide).

31. Incubate the remaining parallel BOD bottles at the test temperature (15-20°C), keep in the dark, and remove from the incubation area at appropriate time intervals, (e.g. after 5, 15 and 28 days as a minimum) and analyse for dissolved oxygen (paragraph 33).

32. Membrane filter (0.2-0.45 μm) or centrifuge, for 15 minutes, samples for specific analyses (optional). Store for up to 48 hours at 2-4°C, or for longer periods at -18°C, if not analysed immediately (if it is known that the test substance will remain unaffected, acidify to pH 2 before storing).

Dissolved oxygen determination

33. Determine the concentration of dissolved oxygen using a chemical or electrochemical method which is recognised nationally or internationally.

DATA AND REPORTING

Treatment of Results

34. Record analytical results on the attached data sheets (Appendix 5).

35. Calculate the BOD as the difference of the oxygen depletion between a blank and a solution of test substance under the conditions of the test. Divide the net oxygen depletion by the concentration (w/v) of the substance in order to express the BOD as mg BOD/mg test substance. The degradation is defined as the ratio of the biochemical oxygen demand to either, preferably, the theoretical oxygen demand (ThOD) or the chemical oxygen demand (COD) and expressed as a percentage (see paragraph 36).

36. Calculate the biodegradation values for each sampling time for both test and reference substances using one or other of the equations:

$$\%\ biodegradation = \frac{mg\ O_2/mg\ tested\ substance}{mg\ ThOD/mg\ tested\ substance} \times 100$$

$$\%\ biodegradation = \frac{mg\ O_2/mg\ tested\ substance}{mg\ COD/mg\ tested\ substance} \times 100$$

where:
ThOD = theoretical oxygen demand (calculation, Appendix 3)
COD = chemical oxygen demand, determined experimentally.

Note: Sometimes the two ways of calculation (percentage of the ThOD or percentage of the COD) do not give the same results; it is preferable to use ThOD, since some substances are not fully oxidised in the COD test.

37. Illustrate the course of the degradation test graphically in a diagram (see example in section on "Validity and interpretation of results". If there are sufficient data, calculate the lag phase (t_L) and the time (t_{50}) to reach 50 per cent removal from the end of the lag phase from the biodegradation curve.

38. If specific analysis is used (optional), state the percentage of primary degradation as the percentage of specific substance removal within the test period (corrected for analytical blanks).

Test Report

39. The test report must contain the following information:

Test substance:
- physical nature and, where relevant, physicochemical properties;
- identification data.

Test conditions:

- location and description of the sampling site: pollutional and nutrient status (colony count, nitrate, ammonium, phosphate if appropriate);
- characteristics of the sample (date of sampling, depth, appearance, temperature, salinity, DOC (optional), delay between collection and use in the test);
- method used (if any) for ageing of the seawater;
- method used for pre-treatment (filtration/sedimentation) of the seawater;
- method used for the COD determination (if performed);
- method used for the oxygen measurements;
- dispersion procedure for substances which are poorly soluble under the test conditions;
- method used for determining the number of heterotrophs in the seawater (plate count method or alternative procedure);
- method used for determining DOC in seawater (optional);
- method used for specific analysis (optional);
- other optional methods used to characterise the seawater (ATP measurements, etc.).

Results:

- analytical data reported on a data sheet (as attached, Appendix 5);
- the course of the degradation test represented graphically in a diagram showing the lag phase, (t_L), slope and time (starting from the end of the lag phase) to reach 50 per cent of the final oxygen uptake caused by oxidation of the test substance (t_{50}). The lag phase may be estimated graphically as shown in the attached figure, or conveniently taken as the time needed for 10 per cent degradation;
- per cent degradation measured after 28 days.

Discussion of results.

Validity and interpretation of results

40. The blank respiration should not exceed 30 per cent of the oxygen in the test bottle. If it is not possible to meet this criterion using freshly collected seawater, the seawater must be aged (stabilized) before use.

41. The possibility that nitrogen-containing substances may affect the results should be considered.

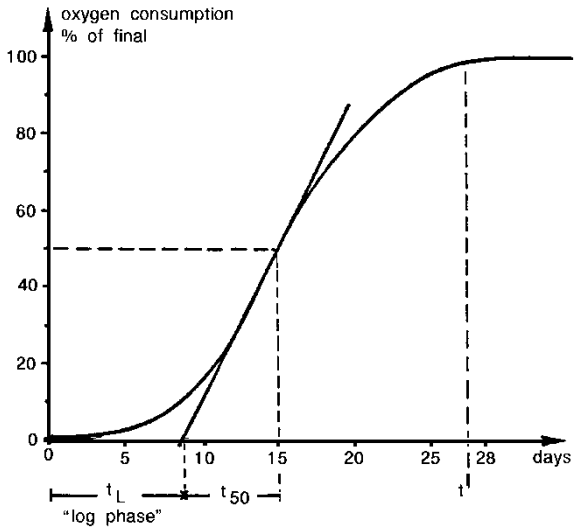
42. Results obtained with the reference substances sodium benzoate and aniline should be comparable to the results obtained in the ring-test (3) (paragraph 9). If results obtained with reference substances are atypical, the test should be repeated using another seawater sample.

43. The test substance can be considered to be inhibitory to bacteria (at the concentration used) if the BOD of the mixture of reference and test substances is less than the sum of the BOD of the separate solutions of the two substances.

44. Owing to the relatively high test concentrations as compared with most natural systems, and consequently an unfavourable ratio between the concentrations of test substance and other carbon sources, the method is to be regarded as a preliminary test

which can be used to indicate whether or not a substance is easily biodegradable. Accordingly, a low result does not necessarily mean that the test substance is not biodegradable in marine environments, but indicates that more work will be necessary in order for this to be established.

An example of a theoretical degradation experiment illustrating a feasible way of estimating the values of t_L (length of "lag phase") and t_{50}, time interval (starting at tL), needed to reach 50% of the final oxygen uptake caused by oxidation of the test substance, is given below:



LITERATURE

(1) de Kreuk J.F. and Hanstveit A.O. (1981). Determination of the biodegradability of the organic fraction of chemical wastes. Chemosphere, 10 (6); 561-573.

(2) Chapter C.4-B of this Annex: Determination of 'Ready' Biodegradability Part III Modified OECD Screening Test

(3) Nyholm N. and Kristensen P. (1987). Screening Test Methods for Assessment of Biodegradability of Chemical Substances in Seawater. Final Report of the ring test programme 1984-1985, March 1987, Commission of the European Communities.

(4) Chapter C.11 of this Annex: Biodegradation — Activated Sludge, Respiration Inhibition Test.

(5) Chapter C.4-E of this Annex: Determination of 'Ready' Biodegradability, Part VI. Closed Bottle Test.

Appendix 1

DETERMINATION OF ORGANIC CARBON IN SEAWATER

SHAKE FLASK METHOD

For the determination of organic carbon of a water sample, the organic compounds in the sample are oxidized to carbon dioxide using generally one of the following three techniques:

- wet-oxidation by persulphate/UV-irradiation;
- wet-oxidation by persulfate/elevated temperature (116-130°C);
- combustion.

Evolved CO_2 is quantified employing infra-red spectrometry or titrimetry. Alternatively, CO_2 is reduced to methane, which is quantified on a flame ionization detector (FID).

The persulfate/UV-method is commonly used for the analysis of "clean" water with low content of particulate matter. The latter two methods can be applied to most kinds of water samples, the persulfate/elevated temperature-oxidation being most suitable for low-level samples, and the combustion technique being applicable for samples with non-volatile organic carbon (NVOC) content well above 1 mg C/l.

Interferences

All three methods are dependent on eliminating or compensating for inorganic carbon (IC) present in the sample. Purging of CO_2 from the acidified sample is the most frequently used method to eliminate the IC, although this also results in a loss of volatile organic compounds (1). The complete elimination or compensation of IC must be ensured for each sample matrix, and volatile organic carbon (VOC) must be determined in addition to NVOC dependent on the sample type.

High chloride concentrations result in decreased oxidation efficiency using the persulfate/UV-method (2). Application of an oxidation reagent modified by the addition of mercury (II) nitrate may, however, remove this interference. It is recommended that the maximum tolerable sample volume be used to evaluate each type of chloride-containing sample. High salt concentrations in sample analysed using the combustion method can cause salt coating of the catalyst and excessive corrosion of the combustion tube. Precautions should be taken according to the manufacturer's manual.

Highly turbid samples as well as samples containing particulate matter may be incompletely oxidized when employing the persulfate/UV-method.

An example of a suitable method

Non-volatile organic carbon is determined by oxidation with persulfate/UV-irradiation and subsequent quantification of evolved CO_2 employing non-dispersive infra-red

spectrometry.

The oxidation reagent is modified in accordance with the suggestions given in (2) as described in the manufacturer's manual:

a) 8.2 g $HgCl_2$ and 9.6 g $Hg(NO_3)_2 \cdot H_2O$ are dissolved in several hundred millilitres of low carbon concentration reagent water.

b) 20 g $K_2S_2O_8$ are dissolved in the mercuric salt solution.

c) 5 ml HNO_3 (conc.) are added to the mixture.

d) the reagent is diluted to 1000 ml.

The interference from chloride is removed using a 40 µl sample volume for 10 per cent chloride and 200 µl sample volume for 1.9 per cent chloride. Samples of high chloride concentrations and/or larger sample volumes can be analysed according to this method provided that build-up of chloride in the oxidation vessel is prevented. Determination of volatile organic carbon can subsequently be performed, if relevant, for the sample type in question.

LITERATURE

(1) ISO, Water quality - determination of total organic carbon. Draft International Standard ISO/DIS 8245, January 16, 1986.

(2) American Public Health Association, Standard Methods for the Estimation of Water and Wastewater. American Water Works Association & Water Pollution Control Federation, 16th edition, 1985.

Also of interest (gives a description of an autoanalysis system):

(3) Schreurs W. (1978). An automated colorimetric method for the determination of dissolved organic carbon in seawater by UV destruction. Hydrobiological Bulletin 12, 137-142.

Appendix 2

BIODEGRADATION IN SEAWATER

SHAKE FLASK METHOD

DATA SHEET

1. **LABORATORY:**

2. **DATE AT START OF TEST:**

3. **TEST SUBSTANCE:**

Name:

Stock solution concentration: mg/l as substance

Initial concentration in medium, t_o: mg/l as substance

: mg DOC/l

4. **SEAWATER:**

Source:

Date of collection:

Depth of collection:

Appearance at time of collection (e.g. turbid, etc.):

Salinity at collection: ‰

Temperature at collection: oC

DOC "x" hours after collection: mg/l

Pretreatment prior to testing (e.g. filtration, sedimentation, ageing, etc.):

Microbial colony count - original sample: colonies/ml

- at start of test: colonies/ml

Other characteristics:

5. **CARBON DETERMINATIONS:**

Carbon analyser:

	Flask no.		DOC after n days (mg/l)				
			0	n_1	n_2	n_3	n_x
Test: nutrient-fortified seawater with test substance	1	a_1					
		a_2					
		mean, $C_{a(t)}$					
	2	b_1					
		b_2					
		mean, $C_{b(t)}$					
Blank: nutrient-fortified seawater without test substance	1	c_1					
		c_2					
		mean, $C_{c(t)}$					
	2	d_1					
		d_2					
		mean, $C_{d(t)}$					
	mean, $C_{bl(t)}=\frac{C_{c(t)}+C_{d(t)}}{2}$						

6. **EVALUATION OF RAW DATA:**

Flask No.	Calculation of results	% Degradation after n days				
		0	n_1	n_2	n_3	n_x
1	$D_1 = 1 - \frac{C_{a(t)} - C_{bl(t)}}{C_0 - C_{bl(0)}} \times 100$	0				
2	$D_2 = 1 - \frac{C_{b(t)} - C_{bl(t)}}{C_0 - C_{bl(0)}} \times 100$	0				
Mean (*)	$D_t = \frac{D_1 + D_2}{2}$	0				

* D_1 and D_2 should not be averaged if there is a considerable difference.

Note: Similar formats may be used when degradation is followed by specific analysis and for the reference substance and toxicity controls.

7. **ABIOTIC DEGRADATION (optional)**

	Time (days)	
	0	t
DOC conc. (mg/l) in sterile control	$C_{s(o)}$	$C_{s(t)}$

$$\%\ abiotic\ degradation = \frac{C_{s(0)} - C_{s(t)}}{C_{s(o)}} x100$$

Appendix 3

CALCULATION OF THE THEORETICAL BIOCHEMICAL OXYGEN DEMAND

CLOSED BOTTLE METHOD

The ThOD of the substance $C_cH_hCl_{cl}N_nNa_{na}O_oP_pS_s$ of the molecular weight MW is calculated according to:

$$ThOD_{NH3} = \frac{16[2c + \frac{1}{2}(h - cl - 3n) + 3s + \frac{5}{2^p} + \frac{1}{2^{na}} - o]}{MW}$$

This calculation implies that C is mineralised to CO_2, H to H_2O, P to P_2O_5 and Na to Na_2O. Halogen is eliminated as hydrogen halide and nitrogen as ammonia.

Example:

Glucose $C_6H_{12}O_6$, MW = 180

$$ThOD = \frac{16(2x6 + \frac{1}{2}x12 - 6)}{180} = 1.07\ mg\ O_2/mg\ glucose$$

Molecular weights of salts other than those of the alkali metals are calculated on the assumption that the salts have been hydrolysed.

Sulphur is assumed to be oxidised to the state of +6.

Example:

Sodium n-dodecylbenzenesulphonate $C_{18}H_{29}SO_3Na$, MW = 348

$$ThOD = \frac{16(36 + \frac{29}{2} + 3 + \frac{1}{2} - 3)}{348} = 2.34\ mg\ O_2/mg\ substance$$

In the case of nitrogen-containing substances the nitrogen may be eliminated as ammonia, nitrite, or nitrate corresponding to different theoretical biochemical oxygen demands.

$$ThOD_{NO2} = \frac{16[2c + \frac{1}{2}(h - cl) + 3s + \frac{3}{2^n} + \frac{5}{2_p} + \frac{1}{2^{na}} - o]}{MW}$$

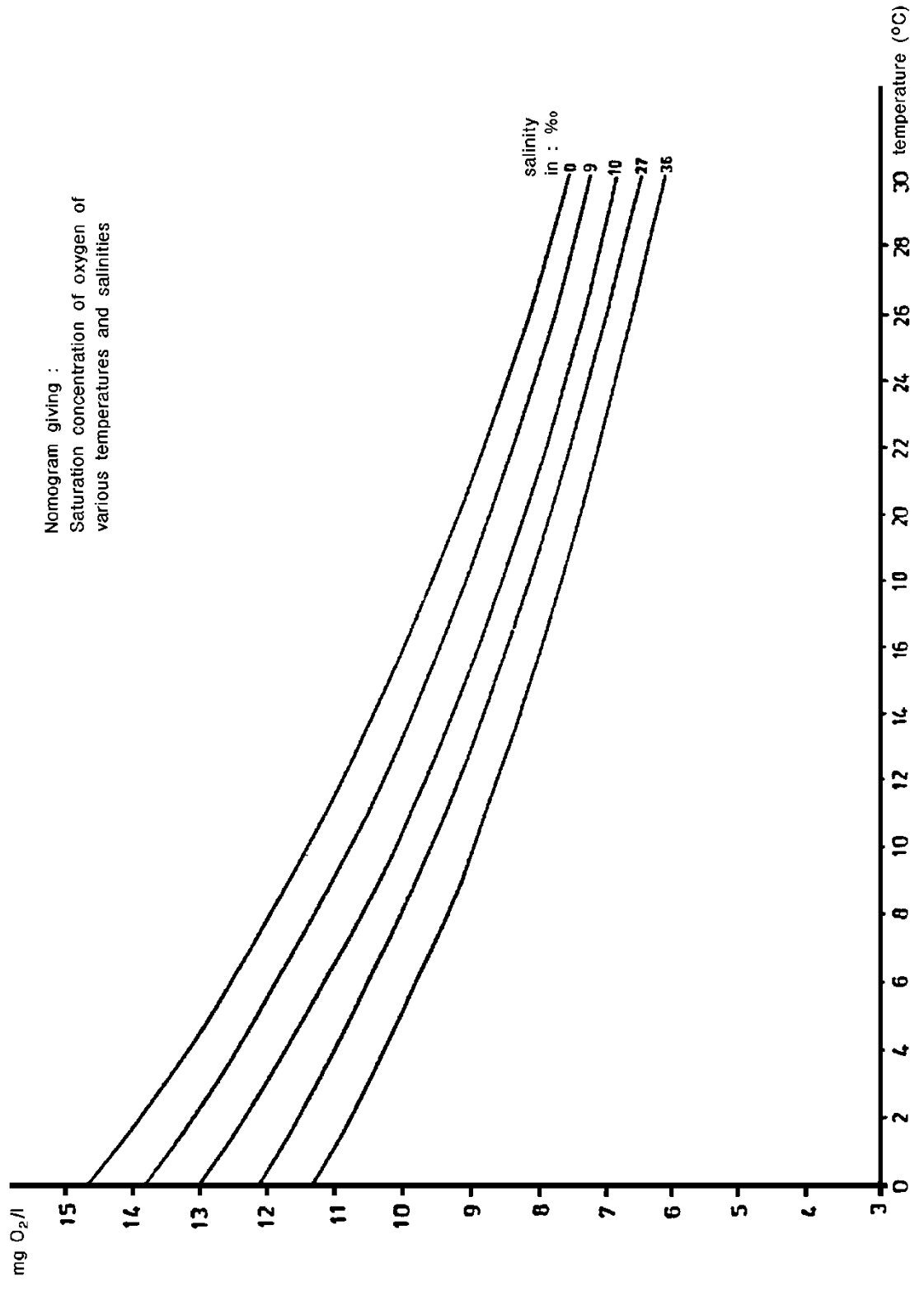
$$ThOD_{NO3} = \frac{16[2c + \frac{1}{2}(h - cl) + 3s + \frac{5}{2^n} + \frac{5}{2^p} + \frac{1}{2^{na}} - o]}{MW}$$

Suppose full nitrate formation had been observed by analysis in the case of a secondary amine:

$(C_{12}H_{25})_2$ NH, MW = 353

$$ThOD_{NO3} = \frac{16(48 + \frac{51}{2} + \frac{5}{2})}{353} = 3.44\ mg\ O_2/mg\ substance$$

Appendix 4


Nomogram giving :
Saturation concentration of oxygen of various temperatures and salinities
salinity in : ‰
0
9
10
27
36
mg O_2/l
15
14
13
12
11
10
9
8
7
6
5
4
3
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30 temperature (°C)

APPENDIX 5

BIODEGRADATION IN SEAWATER

CLOSED BOTTLE METHOD

DATA SHEET

1. **LABORATORY:**
2. **DATE AT START OF TEST:**
3. **TEST SUBSTANCE:**

   Name:

   Stock solution concentration: mg/l

   Initial conc. in seawater medium: mg/l

   ThOD or COD: mg O_2/mg test substance

4. **SEAWATER:**

   Source:

   Date of collection:

   Depth of collection:

   Appearance at time of collection (e.g. turbid, etc.):

   Salinity at collection: ‰

   Temperature at collection: ^{o}C

   DOC "x" hours after collection: mg/l

   Pre-treatment prior to testing (e.g. filtration, sedimentation, ageing, etc.):

   Microbial colony count - original sample: colonies/ml

- at start of test: colonies/ml

Other characteristics:

5. **<u>TEST MEDIUM</u>:**

Temperature after aeration: ^{o}C

O_2 concentration after aeration and

standing before start of test: mg O_2/l

6. **DO DETERMINATION:**

Method: Winkler/electrode

	Flask no.		mg O_2/l after n days			
			0	5	15	28
Test: nutrient - fortified seawater with test substance	1	a_1				
	2	a_2				
	Mean test	$m_t = \frac{a_1 + a_2}{2}$				
Blank: nutrient - fortified seawater, but without test substance	1	c_1				
	2	c_2				
	Mean blank	$m_b = \frac{c_1 + c_2}{2}$				

Note: Similar format may be used for reference substance and toxicity controls.

7. **DO DEPLETION: % DEGRADATION (%D):**

	DO depletion after n days		
	5	15	28
$(m_b - m_t)^{(1)}$			
$\%D = \frac{(m_b - m_t)^{(1)}}{test\ substance\ (mg/l) x ThOD} x100$			

(1) This assumes that $m_{b(o)} = m_{t(o)}$, where
$m_{b(o)}$ = blank value at day 0,
$m_{t(o)}$ = test substance value at day 0.
If $m_{b(o)}$ does not equal $m_{t(o)}$, use $(m_{t(o)} - m_{t(x)}) - (m_{b(o)} - m_{b(x)})$, where
$m_{b(x)}$ = blank value at day x,
$m_{t(x)}$ = test substance value at day x.

C.43. Anaerobic Biodegradability of Organic Substances in Digested Sludge: By Measurement of Gas Production

INTRODUCTION

1. This test method is equivalent to OECD Test Guideline (TG) 311 (2006). There are a number of screening tests for assessing aerobic biodegradability of organic substances (Test methods C.4, C.9, C.10, and C.11 (1) and OECD TG 302C (2)) and the results of applying these have been successfully used to predict the fate of substances in the aerobic environment, particularly in the aerobic stages of waste water treatment. Various proportions of water-insoluble substances, as well as of those which adsorb on to sewage solids, are also dealt with aerobically, since they are present in settled sewage. However, the larger fractions of these substances are bound to the primary settled sludge, which is separated from raw sewage in settlement tanks before the settled, or supernatant, sewage is treated aerobically. The sludge, containing some of the soluble substances in the interstitial liquid, is then passed to heated digesters for anaerobic treatment. As yet there are no tests in this series for assessing anaerobic biodegradability in anaerobic digesters and this test is targeted to fill this gap; it is not necessarily applicable to other anoxic environmental compartments.

2. Respirometric techniques that measure the amounts of gas produced, mainly methane (CH_4) and carbon dioxide (CO_2), under anaerobic conditions have been used successfully for assessing anaerobic biodegradability. Birch et al (3) reviewed these procedures and concluded that the work of Shelton and Tiedje (4), based on earlier studies (5)(6)(7), was the most comprehensive. The method (4), which was further developed by others (8) and has become the American standards (9)(10), did not resolve problems related to the differing solubilities of CO_2 and CH_4 in the test medium and to the calculation of the theoretical gas production of a test substance. The ECETOC report (3) recommended the additional measurement of the dissolved inorganic carbon (DIC) content of the supernatant liquid, which made the technique more widely applicable. The ECETOC method was subjected to an international calibration exercise (or ring test) and became the ISO Standard, ISO 11734 (11).

3. This test method, which is based on ISO 11734 (11), describes a screening method for the evaluation of potential anaerobic biodegradability of organic substances under a specific condition (i.e. in an anaerobic digester at a given time and range of concentration of micro-organisms). Because a diluted sludge is used with a relatively high concentration of test substance and the duration of the test typically is longer than the retention time in anaerobic digesters, the conditions of the test do not necessarily correspond to the conditions in anaerobic digesters, nor is it applicable for the assessment of anaerobic biodegradability of organic substances under different environmental conditions. Sludge is exposed to the test substance for up to 60 days, which is longer than the normal sludge retention time (25 to 30 days) in anaerobic digesters, though at industrial sites retention times may be much longer. Predictions from the results of this test cannot be made as convincingly as they can be made in the case of aerobic biodegradation, since the evidence accrued on the behaviour of test substances in "ready" aerobic tests and in simulation tests and the aerobic environment

is sufficient to be confident that there is a connection; little similar evidence exists for the anaerobic environment. Complete anaerobic biodegradation can be assumed to occur if 75%-80% of theoretical gas production is achieved. The high ratios of substance to biomass used in these tests mean that a substance which passes is more likely to be degraded in an anaerobic digester. Additionally, substances which fail to be converted to gas in the test may not necessarily persist at more environmentally realistic substance-to-biomass ratios. Also, other anaerobic reactions occur by which substances may be at least partially degraded, e.g. by dechlorination, but this test does not detect such reactions. However, by applying specific analytical methods for determining the test substance, its disappearance may be monitored (see paragraphs 6, 30, 44 and 53).

PRINCIPLE OF THE TEST

4. Washed digested sludge[1], containing low (<10 mg/l) concentrations of inorganic carbon (IC), is diluted about ten-fold to a total solids concentration of 1 g/l to 3 g/l and incubated at 35°C ± 2°C in sealed vessels with the test substance at 20 to 100 mg C/l for up to 60 days. Allowance is made for measuring the activity of the sludge by running parallel blank controls with sludge inoculum in the medium but without test substance.

5. The increase in headspace pressure in the vessels resulting from the production of carbon dioxide and methane is measured. Much of the CO_2 produced will be dissolved in the liquid phase or transformed into carbonate or hydrogen carbonate under the conditions of the test. This inorganic carbon is measured at the end of the test.

6. The amount of carbon (inorganic plus methane) resulting from the biodegradation of the test substance is calculated from the net gas production and net IC formation in the liquid phase in excess of blank control values. The extent of biodegradation is calculated from total IC and methane-C produced as a percentage of the measured or calculated amount of carbon added as test substance. The course of biodegradation can be followed by taking intermediate measurements of gas production only. Additionally the primary biodegradation can be determined by specific analyses at the beginning and end of the test.

INFORMATION ON THE TEST SUBSTANCE

7. The purity, water solubility, volatility and adsorption characteristics of the test substance should be known to enable correct interpretation of results to be made. The organic carbon content (% w/w) of the test substance needs to be known either from its

[1] Digested sludge is a mixture of the settled phases of sewage and activated sludge, which have been incubated in an anaerobic digester at about 35°C to reduce biomass and odour problems and to improve the dewater-ability of the sludge. It consists of an association of anaerobic fermentative and methanogenic bacteria producing carbon dioxide and methane (11).

chemical structure or by measurement. For volatile test substances, a measured or calculated Henry's law constant is helpful in deciding whether the test is applicable. Information on the toxicity of the test substance for anaerobic bacteria is useful in selecting an appropriate test concentration, and for interpreting results showing poor biodegradability. It is recommended to include the inhibition control unless it is known that the test substance is not inhibitory to anaerobic microbial activities (see paragraph 21 and ISO 13641-1 (12)).

APPLICABILITY OF THE TEST METHOD

8. The test method may be applied to water-soluble substances; it may also be applied to poorly soluble and insoluble substances, provided that a method of exact dosing is used e.g. see ISO 10634 (13). In general, a case by case decision is necessary for volatile substances. Special steps may have to be taken, for example, not releasing gas during the test.

REFERENCE SUBSTANCES

9. To check the procedure, a reference substance is tested by setting up appropriate vessels in parallel as part of normal test runs. Phenol, sodium benzoate and polyethylene glycol 400 are examples and would be expected to be degraded by more than 60% theoretical gas production (i.e. methane and inorganic carbon) within 60 days (3)(14).

REPRODUCIBILITY OF TEST RESULTS

10. In an international ring test (14) there was good reproducibility in gas pressure measurements between triplicate vessels. The relative standard deviation (coefficient of variation, COV) was mainly below 20 %, although this value often increased to >20% in the presence of toxic substances or towards the end of the 60-d incubation period. Higher deviations were also found in vessels of volume <150 ml. Final pH values of the test media were in the range 6.5-7.0.

11. The following results were obtained in the ring test.

Test substance	Total data n_1	Mean degradation (of total data) (%)	Relative Standard deviation (of total data) (%)	Valid data n2	Mean degradation (of valid data) (%)	Relative Standard deviation (of valid data) (%)	Data >60% degradation in valid tests n_3
Palmitic acid	36	68.7 $\pm$ 30.7	45	27	72.2 $\pm$ 18.8	26	19 = 70%*
Polyethylene	38	79.8 $\pm$ 28.0	35	29	77.7 $\pm$ 17.8	23	24 = 83 %*

Glycol 400							

* Proportion of n_2

12. The coefficients of variation of the mean for all values obtained with palmitic acid and polyethylene glycol 400 were as high as 45% (n = 36) and 35% (n = 38) respectively. When values of <40% and >100% were omitted (the former being assumed to be due to sub-optimal conditions, the latter due to unknown reasons), the COVs were reduced to 26% and 23%, respectively. The proportions of "valid" values attaining at least 60% degradation were 70% for palmitic acid and 83% for polyethylene glycol 400. The proportions of the percentage biodegradation derived from DIC measurements were relatively low but variable. For palmitic acid the range was 0-35%, mean 12%, with COV of 92% and for polyethyleneglycol 400 0-40%, mean 24%, with COV of 54%.

DESCRIPTION OF THE TEST METHOD

Apparatus

13. Usual laboratory equipment and the following are required:

a. Incubator - spark-proof and controlled at 35°C ± 2°C;
b. Pressure-resistant glass test vessels of an appropriate nominal size[1], each fitted with a gas-tight septum, capable of withstanding about 2 bar. The headspace volume should be about 10% to 30% of the total volume. If biogas is released regularly, about 10% headspace volume is appropriate, but if the gas release is made only at the end of the test 30% is appropriate. Glass serum bottles, of nominal volume 125 ml, total volume around 160 ml, sealed with serum septa[2] and crimped aluminium rings are recommended when the pressure is released at each sampling time;
c. Pressure-measuring device[3] adapted to enable measurement and venting of the gas produced, for example, a hand-held precision pressure meter connected to a suitable syringe needle; a 3-way gas-tight valve facilitates the release of excess pressure (Appendix 1). It is necessary to keep the internal volume of the pressure transducer tubing and valve as low as possible, so that errors introduced by neglecting the volume of the equipment are insignificant;

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[1] The recommended size is 0.1 litre to 1 litre.

[2] The use of gas-tight silicone septa is recommended. It is further recommended that the gas-tightness of caps, especially butyl rubber septa, be tested because several commercially available septa are not sufficiently gas-tight against methane and some septa do not stay tight when they are pierced with a needle under the conditions of the test.

[3] The device should be used and calibrated at regular intervals, according to the manufacturer's instructions. If a pressure-meter of the prescribed quality is used e.g. capsulated with a steel membrane, no calibration is necessary in the laboratory. The accuracy of the calibration can be checked at the laboratory with a one-point measurement at 1 x 10^5 Pa against a pressure-meter with a mechanical display. When this point is measured correctly, the linearity will also be unaltered. If other measurement devices are used (without certified calibration by the manufacturer), calibration is recommended over the total range at regular intervals.

Note – The pressure readings are used directly to calculate the amount of carbon produced in the headspace (paragraphs 42 to 44). Alternatively, the pressure readings may be converted to volumes (at 35°C, atmospheric pressure) of gas produced using a conversion graph. This graph is constructed from data obtained by injecting known volumes of nitrogen gas into a series of test vessels (e.g. serum bottles) at 35° +/- 2°C and recording the resulting stabilised pressure readings (See Appendix 2). The calculation is shown in the Note in paragraph 44.

Warning – Take care to avoid needle-stick injuries when using micro-syringes.

d. Carbon analyser, suitable for the direct determination of inorganic carbon in the range of 1 mg/l to 200 mg/l;
e. Syringes of high precision for gaseous and liquid samples;
f. Magnetic stirrers and followers (optional);
g. Glove box (recommended).

Reagents

14. Use analytical grade reagents throughout.

Water

15. Distilled or deionised water (de-oxygenated by sparging with nitrogen gas containing less than 5 µl/l oxygen), containing less than 2 mg/l dissolved organic carbon (DOC).

Test medium

16. Prepare the dilution medium to contain the following constituents at the stated amounts;

Anhydrous potassium dihydrogen phosphate (KH_2PO_4)	0.27 g
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$))	1.12 g
Ammonium chloride (NH_4Cl)	0.53 g
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	0.075g
Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	0.10 g
Iron (II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$)	0.02 g
Resazurin (oxygen indicator)	0.001g
Sodium sulphide nonahydrate ($Na_2S \cdot 9H_2O$)	0.10 g
Stock solution of trace elements (optional, paragraph 18)	10 ml
Add de-oxygenated water (paragraph 15)	to 1 litre

Note: Freshly supplied sodium sulphide should be used or it should be washed and dried before use, to ensure sufficient reductive capacity. The test may be performed without using a glove box (see paragraph 26). In this case, the final concentration of sodium sulphide in the medium should be increased to 0.20 g of $Na_2S \cdot 9H_2O$ per litre. Sodium sulphide may also be added from an appropriate anaerobic stock solution through the septum of the closed test vessels as this procedure will decrease the risk of oxidation. Sodium sulphide may be replaced by titanium (III) citrate, which is added through the septum of closed test vessels at a final concentration of 0.8 to 1.0 mmol/l. Titanium (III) citrate is a highly effective and low-toxicity reducing agent, which is prepared as follows: Dissolve 2.94 g of trisodium citrate dihydrate in 50 ml of de-oxygenated water (to result in a solution of 200 mmol/l) and add 5 ml of a 15% (w/v) titanium (III) chloride solution. Neutralise to pH 7 $\pm$ 0.2 with mineral alkali and dispense to an appropriate vessel under a stream of nitrogen. The concentration of titanium (III) citrate in this stock solution is 164 mmol/l.

17. Mix the components of the test medium except the reducing agent (sodium sulphide titanium citrate) and sparge the solution with nitrogen gas for about 20 min immediately before use to remove oxygen. Then add the appropriate volume of freshly prepared solution of the reducing agent (prepared in de-oxygenated water) just before use of the medium. Adjust the pH of the medium, if necessary, with dilute mineral acid or alkali to 7 $\pm$ 0.2.

Stock solution of trace elements (optional)

18. It is recommended that the test medium should contain the following trace elements to improve anaerobic degradation processes, especially if low concentrations (e.g. 1g/l) of inoculum are used (11).

Manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$)	50 mg
Boric acid (H_3BO_3)	5 mg
Zinc chloride ($ZnCl_2$)	5 mg
Copper (II) chloride ($CuCl_2$)	3 mg
Disodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$)	1 mg
Cobalt chloride hexahydrate ($CoCl_2 \cdot 6H_2O$)	100 mg
Nickel chloride hexahydrate ($NiCl_2 \cdot 6H_2O$)	10 mg
Disodium selenite (Na_2SeO_3)	5 mg
Add de-oxygenated water (paragraph 15)	to 1 litre

Test substance

19. Add the test substance as a stock solution, suspension, emulsion, or directly as solid or liquid, or as absorbed on to glass-fibre filter to give a concentration of no more than 100 mg/l organic carbon. If stock solutions are used, prepare a suitable solution with water (paragraph 15) (previously de-oxygenated by sparging with nitrogen gas) of such a strength that the volume added is less than 5% of the total volume of reaction mixture. Adjust the pH of the stock solution to pH 7 $\pm$ 0.2 if necessary. For test substances which are insufficiently soluble in water, consult ISO 10634 (13). If a

solvent is used, prepare an additional control, with the solvent only added to the inoculated medium. Organic solvents which are known to inhibit methane production, such as chloroform and carbon tetrachloride, should be avoided.

Warning - Handle with care toxic test substances, and those whose properties are not known.

Reference substances

20. Reference substances such as sodium benzoate, phenol and polyethylene glycol 400 have been used successfully to check the procedure, being biodegraded by more than 60% within 60 days. Prepare a stock solution (in de-oxygenated water) of the chosen reference substance in the same way as for the test substance and adjust to pH 7 ± 0.2 if necessary.

Inhibition control (conditional)

21. In order to obtain information on the toxicity of the test substance to anaerobic micro-organisms to find the most appropriate test concentration, add the test substance and reference substance to a vessel containing the test medium (see paragraph 16), each at the same concentrations as added, respectively (see paragraphs 19 and 20 and see also ISO 13641-1 (12)).

Digested sludge

22. Collect digested sludge from a digester at a waste water treatment plant which treats predominantly domestic sewage. The sludge should be fully characterised and its background information should be reported (see paragraph 54). If use of adapted inoculum is intended, digested sludge from an industrial sewage treatment plant may be considered. Use wide-necked bottles constructed from high-density polyethylene or a similar material, which can expand, for the collection of the digested sludge. Add sludge to within about 1cm of the top of the bottles and seal tightly, preferably with a safety valve. After transport to the laboratory, the collected sludge may be used directly or placed in a laboratory-scale digester. Release excess biogas by opening bottles of sludge carefully. Alternatively, laboratory-grown anaerobic sludge may be used as a source of inoculum but its spectrum of activity may have been impaired.

Warning - Digested sludge produces flammable gases which present fire and explosion risks: it also contains potentially pathogenic organisms, so take appropriate precautions when handling sludge. For safety reasons, do not use glass vessels for collecting sludge.

23. In order to reduce background gas production and to decrease the influence of the blank controls, pre-digestion of the sludge may be considered. If pre-digestion is required, the sludge should be allowed to digest without the addition of any nutrients or substrates at 35°C ± 2°C for up to 7 days. It has been found that pre-digestion for about 5 days usually gives an optimal decrease in gas production of the blank without unacceptable increases in either lag or incubation periods during the test phase or loss of activity towards a small number of substances tested.

24. For test substances which are, or are expected to be, poorly biodegradable, consider pre-exposure of the sludge to the test substance to obtain an inoculum which is better adapted. In such a case, add the test substance at an organic carbon concentration of 5 mg/l to 20 mg/l to the digested sludge and incubated for up to 2 weeks. Wash the pre-exposed sludge carefully before use (see paragraph 25) and indicate in the test report the conditions of the pre-exposure.

Inoculum

25. Wash the sludge (see paragraphs 22 to 24) just prior to use, to reduce the IC concentration to less than 10 mg/l in the final test suspension. Centrifuge the sludge in sealed tubes (e.g. 3 000 g during 5 min) and discharge the supernatant. Suspend the resulting pellet in de-oxygenated medium (paragraphs 16 and 17), re-centrifuge the suspension and discharge the supernatant liquid. If the IC has not been sufficiently lowered, the washing procedure of the sludge could be repeated twice as a maximum. This does not appear to affect the micro-organisms adversely. Finally, suspend the pellet in the requisite volume of test medium and determine the concentration of total solids [e.g. ISO 11923 (15)]. The final concentration of total solids in the test vessels should be in the range of 1 g/l to 3 g/l (or about 10% of that in undiluted digested sludge). Conduct the above operations in such a way that the sludge has minimal contact with oxygen (e.g. use a nitrogen atmosphere).

TEST PROCEDURE

26. Perform the following initial procedures using techniques to keep the contact between digested sludge and oxygen as low as practicable, for example, it may be necessary to work within a glove box in an atmosphere of nitrogen and/or purge the bottles with nitrogen (4).

Preparation of test and control assays

27. Prepare at least triplicate test vessels (see paragraph 13-b) for the test substance, blank controls, reference substance, inhibition controls (conditional) and pressure control chambers (optional procedure) (see paragraphs 7, 19 to 21). Additional vessels for the purpose of evaluating primary biodegradation using test substance specific analyses may also be prepared. The same set of blank controls may be used for several test substances in the same test as long as the headspace volumes are consistent.

28. Prepare the diluted inoculum before adding it to the vessels e.g. by the means of a wide-mouthed pipette. Add aliquots of well-mixed inoculum (paragraph 25) so that the concentration of total solids is the same in all vessels (between 1 g/l and 3 g/l). Add stock solutions of the test and reference substance after adjustment to pH 7 ± 0.2, if necessary. The test substance and the reference substance should be added using the most appropriate route of administration (paragraph 19).

29. The test concentration of organic carbon should normally be between 20 and 100 mg/l (paragraph 4). If the test substance is toxic, the test concentration should be reduced to 20 mg C/l, or even less if only primary biodegradation with specific analyses is to be

measured. It should be noted that the variability of the test results increases at lower test concentrations.

30. For blank vessels, add an equivalent amount of the carrier used to dose the test substance instead of a stock solution, suspension or emulsion. If the test substance was administered using glass fibre filters or organic solvents, add to the blanks a filter or an equivalent volume solvent that has been evaporated. Prepare an extra replicate with test substance for the measurement of the pH value. Adjust the pH to 7 ± 0.2, if necessary, with small amounts of dilute mineral acid or alkali. The same amounts of neutralising agents should be added to all the test vessels. These additions should not have to be made since the pH value of the stock solutions of the test substance and reference substance have already been adjusted (see paragraphs 19 and 20). If primary biodegradation is to be measured, an appropriate sample should be taken from the pH-control vessel, or from an additional test vessel, and the test substance concentration should be measured using specific analyses. Covered magnets may be added to all the vessels if the reaction mixtures are to be stirred (optional).

31. Ensure that the total volume of liquid V_l and the volume of headspace V_h are the same in all vessels; note and record the values of V_l and V_h. Each vessel should be sealed with a gas septum and transferred from the glove box (see paragraph 26) into the incubator (see paragraph 13-a).

Insoluble test substances

32. Add weighed amounts of substances, which are poorly soluble in water, directly to the prepared vessels. When the use of a solvent is necessary (see paragraph 19), transfer the test substance solution or suspension into the empty vessels. Where possible, evaporate the solvent by passing nitrogen gas through the vessels and then add the other ingredients, namely, diluted sludge (paragraph 25), and de-oxygenated water as required. An additional solvent control should also be prepared (see paragraph 19). For other methods of adding insoluble substances, ISO 10634 (13) can be consulted. Liquid test substances may be dosed with a syringe into the completely prepared sealed vessels, if it is expected that the initial pH will not exceed 7 ± 1, otherwise dose as described above (see paragraph 19).

Incubation and gas pressure measurements

33. Incubate the prepared vessels at $35°C \pm 2°C$ for about 1h to allow equilibration and release excess gas to the atmosphere, for example, by shaking each vessel in turn, inserting the needle of the pressure meter (paragraph 13-c) through the seal and opening the valve until the pressure meter reads zero. If at this stage, or when making intermediate measurements, the headspace pressure is less than atmospheric, nitrogen gas should be introduced to re-establish atmospheric pressure. Close the valve (see paragraph 13-c) and continue to incubate in the dark, ensuring that all parts of the vessels are maintained at the digestion temperature. Observe the vessels after incubation for 24 to 48h. Reject vessels if the contents of the vessels show a distinct pink coloration in the supernatant liquid, i.e. if Resazurin (see paragraph 16) has changed colour indicating the presence of oxygen (see paragraph 50). While small amounts of oxygen may be tolerated by the system, higher concentrations can

seriously inhibit the course of anaerobic biodegradation. The rejection of the occasional single vessel of a set of triplicates may be accepted, but the incidence of more failures than this must lead to an investigation of the experimental procedures as well as the repeating of the test.

34. Carefully mix the contents of each vessel by stirring or by shaking for a few minutes at least 2 or 3 times per week and soon before each pressure measurement. Shaking re-suspends the inoculum and ensures gaseous equilibrium. All pressure measurements should be taken quickly, since the test vessels could be subject to lowering of temperature, leading to false readings. While measuring pressure the whole test vessel including the headspace should be maintained at the digestion temperature. Measure the gas pressure, for example, by inserting through the septum the syringe needle (paragraph 13-c) connected to the pressure-monitoring meter. Care should be taken to prevent entry of water into the syringe needle; if this occurs the wet parts should be dried and a new needle fitted. The pressure should be measured in millibars (see paragraph 42). The gas pressure in the vessels may be measured periodically e.g. weekly, and optionally the excess gas is released to the atmosphere. Alternatively the pressure is measured only at the end of the test to determine the amount of biogas produced.

35. It is recommended that intermediate readings of gas pressure be made, since pressure increase provides guidance as to when the test may be terminated and allows the kinetics to be followed (see paragraph 6).

36. Normally end the test after an incubation period of 60 days unless the biodegradation curve obtained from the pressure measurements has reached the plateau phase before then; that is the phase in which the maximal degradation has been reached and the biodegradation curve has levelled out. If the plateau value is less than 60 % interpretation is problematic because it indicates that only part of the molecule has been mineralised or that an error has been made. If at the end of the normal incubation period, gas is being produced but a plateau phase is obviously not reached, then it should be considered to prolong the test to check whether the plateau (> 60%) will be reached.

Measurement of inorganic carbon

37. At the end of the test after the last measurement of gas pressure, allow the sludge to settle. Open each vessel in turn and immediately take a sample for the determination of the concentration (mg/l) of inorganic carbon (IC) in the supernatant liquor. Neither centrifugation nor filtration should be applied to the supernatant liquor, since there would be an unacceptable loss of dissolved carbon dioxide. If the liquor cannot be analysed on being sampled, store it in a sealed vial, without headspace and cooled to about 4°C for up to 2 days. After the IC measurement, measure and record the pH value.

38. Alternatively, the IC in the supernatant may be determined indirectly by release of the dissolved IC as carbon dioxide that can be measured in the headspace. Following the last measurement of gas pressure, adjust the pressure in each of the test vessels to atmospheric pressure. Acidify the contents of each vessel to approximately pH 1 by

adding of concentrated mineral acid (e.g. H_2SO_4) through the septum of the sealed vessels. Incubate the shaken vessels at 35°C ± 2°C for approximately 24 hours and measure the gas pressure resulting from the evolved carbon dioxide by using the pressure meter.

39. Make similar readings for the corresponding blank, reference substance and, if included, inhibition control vessels (see paragraph 21).

40. In some cases, especially if the same control vessels are used for several test substances, measurements of intermediate IC concentrations in test and control vessels should be considered, as appropriate. In this case, a sufficient number of vessels should be prepared for all the intermediate measurements. This proceeding is preferred to taking all samples from one vessel only. The latter can only be done if the required volume for DIC analysis is not deemed to be too high. The DIC measurement should be made after measuring the gas pressure without release of excess gas as described below:

- take as small a volume as possible of supernatant samples with a syringe through the septum without opening the vessels and IC in the sample is determined;
- after having taken the sample the excess gas is released, or not;
- it should be taken into account that even a small decrease in the supernatant volume (e.g. about 1%) can yield a significant increase in the headspace gas volume (V_h);
- the equations (see paragraph 44) are corrected by increasing V_h in equation 3, as necessary.

Specific analyses

41. If primary anaerobic degradation (see paragraph 30) is to be determined, take an appropriate volume of sample for specific analyses at the beginning and at the end of the test from the vessels containing the test substance. If this is done, note the volumes of headspace (V_h) and of the liquid (V_l) will be changed and take this into account when calculating the results of gas production. Alternatively samples may be taken for specific analyses from additional mixtures previously set up for the purpose (paragraph 30).

DATA AND REPORTING

Treatment of results

42. For practical reasons, the pressure of the gas is measured in millibars (1 mbar = 1h Pa = 10^2 Pa; 1 Pa = 1 N/m^2), the volume in litres and temperature in degrees Celsius.

Carbon in the headspace

43. Since 1 mol of methane and 1 mol carbon dioxide each contain 12 g of carbon, the mass of carbon in a given volume of evolved gas may be expressed as:

$$m = 12 \times 10^3 \times n \qquad \text{Equation [1]}$$

where:
m = mass of carbon (mg) in a given volume of evolved gas;
12 = relative atomic mass of carbon;
n = number of moles of gas in the given volume.

If a gas other than methane or carbon dioxide (e.g. N_2O) is generated in considerable amounts, the formula [1] should be amended in order to describe the possibility of effects by gases generated.

44. From the gas laws n may be expressed as:

$$n = \frac{pV}{RT} \qquad \text{Equation [2]}$$

where:
p = pressure of the gas (Pascals);
V = volume of the gas (m^3);
R = molar gas constant [8.314J/(mol K)];
T = incubation temperature (Kelvins).

By combination of equations [1] and [2] and rationalising to allow for blank control production of gas:

$$m_h = \frac{12000 \times 0.1(\Delta p \cdot V_h)}{RT} \qquad \text{Equation [3]}$$

where:
m_h = mass of net carbon produced as gas in the headspace (mg);
Δp = mean of the difference between initial and final pressures in the test vessels minus the corresponding mean in the blank vessels (millibars);
V_h = volume of headspace in the vessel (l);
0.1 = conversion for both newtons/m^2 to millibars and m^3 to litres.

Equation [4] should be used for the normal incubation temperature of 35°C (308 K):

$$m_h = 0.468(\Delta p \cdot V_h) \qquad \text{Equation [4]}$$

Note: Alternative volume calculation. Pressure meter readings are converted to ml of gas produced using the standard curve generated by plotting volume (ml) injected versus meter reading (Appendix 2). The number of moles (n) of gas in the headspace of each vessel is calculated by dividing the cumulative gas production (ml) by 25286 ml/mole, which is the volume occupied by one mole of gas at 35 °C and standard atmospheric pressure. Since 1 mole of CH_4 and 1 mole of CO_2 each contain 12 g of carbon, the amount of carbon (mg) in the headspace (m_h) is given by Equation [5]:

$$m_h = 12 \times 10^3 \times n \qquad \text{Equation [5]}$$

Rationalising to allow for blank control production of gas:

$$m_h = \frac{12000 \times \Delta V}{25286} = 0.475 \Delta V \qquad \text{Equation [6]}$$

where:
m_h = mass of net carbon produced as gas in the headspace (mg);
ΔV = mean of the difference between volume of gas produced in headspace in the test vessels and blank control vessels;
25286 = volume occupied by 1 mole gas at 35°C, 1 atmosphere.

45. The course of biodegradation can be followed by plotting the cumulated pressure increase Δp (millibars) against time, if appropriate. From this curve, identify and record the lag phase (days). The lag phase is the time from the start of the test until significant degradation starts (for example see Appendix 3). If intermediate samples of supernatant were taken and analysed (see paragraphs 40, 46 and 47), then the total C produced (in gas plus that in liquid) may be plotted instead of only the cumulative pressure.

Carbon in the liquid

46. The amount of methane in the liquid is ignored since its solubility in water is known to be very low. Calculate the mass of inorganic carbon in the liquid of the test vessels

using equation [7]:

$$m_l = C_{net} \times V_l \qquad \text{Equation [7]}$$

where:
m_l = mass of inorganic carbon in the liquid (mg);
C_{net} = concentration of inorganic carbon in the test vessels minus that in the control vessels at the end of the test (mg/l);
V_l = volume of liquid in the vessel (l).

Total gasified carbon

47. Calculate the total mass of gasified carbon in the vessel using equation [8]:

$$m_t = m_h + m_l \qquad \text{Equation [8]}$$

where:
m_t = total mass of gasified carbon (mg);
m_h and m_l are as defined above.

Carbon of test substance

48. Calculate the mass of carbon in the test vessels derived from the added test substance using equation [9]:

$$m_v = C_c \times V_l \qquad \text{Equation [9]}$$

where:
m_v = mass of test substance carbon (mg);
C_c = concentration of test substance carbon in the test vessel (mg/l)
V_l = volume of liquid in the test vessel (l).

Extent of biodegradation

49. Calculate the percentage biodegradation from headspace gas using equation [10] and the total percentage biodegradation using equation [11]:

$$D_h = \left(m_h / m_v\right) \times 100 \qquad \text{Equation [10]}$$

$$D_t = \left(m_t / m_v\right) \times 100 \qquad \text{Equation [11]}$$

where:
D_h = biodegradation from headspace gas (%);
D_t = total biodegradation (%);
m_h, m_v and m_t are as defined above.

The degree of primary biodegradation is calculated from the (optional) measurements of the concentration of the test substance at the beginning and end of incubation, using equation [12]:

$$D_p = \left(1 - S_e / S_i\right) \times 100 \qquad \text{Equation [12]}$$

where:
D_p = primary degradation of test substance (%);
S_i = initial concentration of test substance (mg/l);
S_e = concentration of test substance at end (mg/l).

If the method of analysis indicates significant concentrations of the test substance in the unamended anaerobic sludge inoculum, use equation [13]:

$$D_p{}^1 = \left[1 - \left(S_e - S_{eb}\right) / \left(S_i - S_{ib}\right)\right] \times 100 \qquad \text{Equation [13]}$$

where:
$D_p{}^1$ = corrected primary degradation of test substance (%);
S_{ib} = initial 'apparent' concentration of test substance in blank controls (mg/l);
S_{eb} = 'apparent' concentration of test substance in blank controls at end (mg/l).

Validity of results

50. Pressure readings should be used only from vessels that do not show pink coloration (see paragraph 33). Contamination by oxygen is minimised by the use of proper anaerobic handling techniques.

51. It should be considered that the test is valid if the reference substance reaches a plateau that represents more than 60% biodegradation.[1]

52. If the pH at the end of the test has exceeded the range 7 ± 1 and insufficient biodegradation has taken place, repeat the test with increased buffer capacity of the medium.

Inhibition of degradation

53. Gas production in vessels containing both the test substance and reference substance should be at least equal to that in the vessels containing only reference substance; otherwise, inhibition of gas production is indicated. In some cases gas production in vessels containing test substance without reference substance will be lower than that in the blank controls, indicating that the test substance is inhibitory.

Test report

54. The test report must include the following information:

Test substance:
- common name, chemical name, CAS number, structural formula and relevant physical-chemical properties;
- purity (impurities) of test substance.

Test conditions:
- volumes of diluted digester liquor (V_l) and of the headspace (V_h) in the vessel;
- description of the test vessels, the main characteristics of biogas measurement (e.g. type of pressure meter) and of the IC analyser;
- application of test substance and reference substance to test system: test concentration used and any use of solvents;
- details of the inoculum used: name of sewage treatment plant, description of the source of waste water treated (e.g. operating temperature, sludge retention time, predominantly domestic, etc.), concentration, any information necessary to substantiate this and information on any pre-treatment of the inoculum (e.g. pre-digestion, pre-exposure);
- incubation temperature;
- number of replicates.

Results:
- pH and IC values at the end of the test;
- concentration of test substance at the beginning and end of the test, if a specific measurement has been performed;
- all the measured data collected in the test, blank, reference substance and inhibition control vessels, as appropriate (e.g. pressure in millibars, concentration of inorganic carbon (mg/l)) in tabular form (measured data for headspace and liquid should be

[1] This should be re-evaluated if adsorptive and insoluble reference chemicals are included.

reported separately);
- statistical treatment of data, test duration and a diagram of the biodegradation of test substance, reference substance and inhibition control;
- percentage biodegradation of test substance and reference substance;
- reasons for any rejection of the test results;
- discussion of results.

LITERATURE

(1) The following chapters of this Annex:
C.4,Determination of Ready Biodegradability;
C.9, Biodegradation – Zahn-Wellens Test;
C.10, Simulation Test - Aerobic Sewage Treatment:
A: Activated Sludge Units, B: Biofilms
C.11, Biodegradation – Activated sludge respiration inhibition

(2) OECD (2009) Inherent Biodegradability: Modified MITI Test (II), OECD Guideline for Testing of Chemicals, No. 302C, OECD, Paris

(3) Birch, R. R., Biver, C., Campagna, R., Gledhill, W.E., Pagga,U., Steber, J., Reust, H. and Bontinck, W.J. (1989) Screening of chemicals for anaerobic biodegradation. Chemosphere 19, 1527-1550. (Also published as ECETOC Technical Report No. 28, June 1988).

(4) Shelton D.R. and Tiedje, J.M. (1984) General method for determining anaerobic biodegradation potential. Appl. Environ. Mircobiology, 47, 850-857.

(5) Owen, W.F., Stuckey, DC., Healy J.B., Jr, Young L.Y. and McCarty, P.L. (1979) Bioassay for monitoring biochemical methane potential and anaerobic toxicity. Water Res. 13, 485-492.

(6) Healy, J.B.Jr. and Young, L.Y. (1979) Anaerobic biodegradation of eleven aromatic compounds to methane. Appl. Environ. Microbiol. 38, 84-89.

(7) Gledhill, W.E. (1979) Proposed standard practice for the determination of the anaerobic biodegradation of organic chemicals. Working document. Draft 2 no.35.24. American Society for Testing Materials, Philadelphia.

(8) Battersby, N.S. and Wilson, V. (1988) Evaluation of a serum bottle technique for assessing the anaerobic biodegradability of organic chemicals under methanogenic conditions. Chemosphere, 17, 2441-2460.

(9) E1192-92. Standard Test Method for Determining the Anaerobic Biodegradation Potential of Organic Chemicals. ASTM, Philadelphia.

(10) US-EPA (1998) Fate, Transport and Transformation Test Guidelines OPPTS 835.3400 Anaerobic Biodegradability of Organic Chemicals.

(11) International Organization for Standardization (1995) ISO 11 734 Water Quality - Evaluation of the ultimate anaerobic biodegradation of organic compounds in digested sludge - Method by measurement of the biogas production.

(12) International Organization for Standardization (2003) ISO 13 641-1 Water Quality - Determination of inhibition of gas production of anaerobic bacteria - Part 1 General Test.

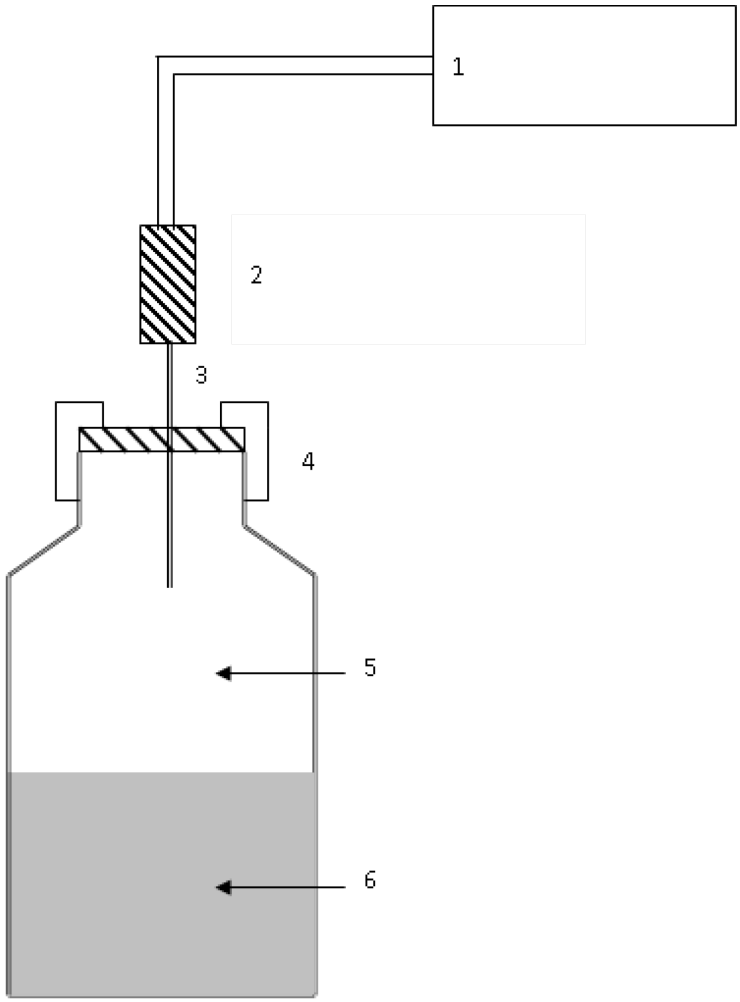
(13) International Organization for Standardization (1995) ISO 10 634 Water Quality - Guidance for the preparation and treatment of poorly water-soluble organic compounds for the subsequent evaluation of their biodegradability in an aqueous medium.

(14) Pagga, U. and Beimborn, D.B., (1993) Anaerobic biodegradation test for organic compounds. Chemosphere, 27, 1499-1509.

(15) International Organization for Standardization (1997) ISO 11 923 Water Quality - Determination of suspended solids by filtration through glass-fibre filters.

APPENDIX 1

EXAMPLE OF AN APPARATUS TO MEASURE BIOGAS PRODUCTION BY GAS PRESSURE



Key:
1- Pressure meter
2- 3-way gas-tight valve
3 - Syringe needle
4 - Gastight seal (crimp cap and septum)
5 - Head space (V_h)
6 - Digested sludge inoculum (V_l)

Test vessels in an environment of 35 °C ± 2 °C

APPENDIX 2

CONVERSION OF THE PRESSURE-METER

The pressure-meter readings may be related to gas volumes by means of a standard curve produced by injecting known volumes of air at 35°C ± 2°C into serum bottles containing a volume of water equal to that of the reaction mixture, V_R:

- Dispense V_R ml aliquots of water, kept at 35°C ± 2°C into five serum bottles. Seal the bottles and place in a water bath at 35 °C for 1 hour to equilibrate;

- Switch on the pressure-meter, allow to stabilise, and adjust to zero;

- Insert the syringe needle through the seal of one of the bottles, open the valve until the pressure meter reads zero and close the valve;

- Repeat the procedure with the remaining bottles;

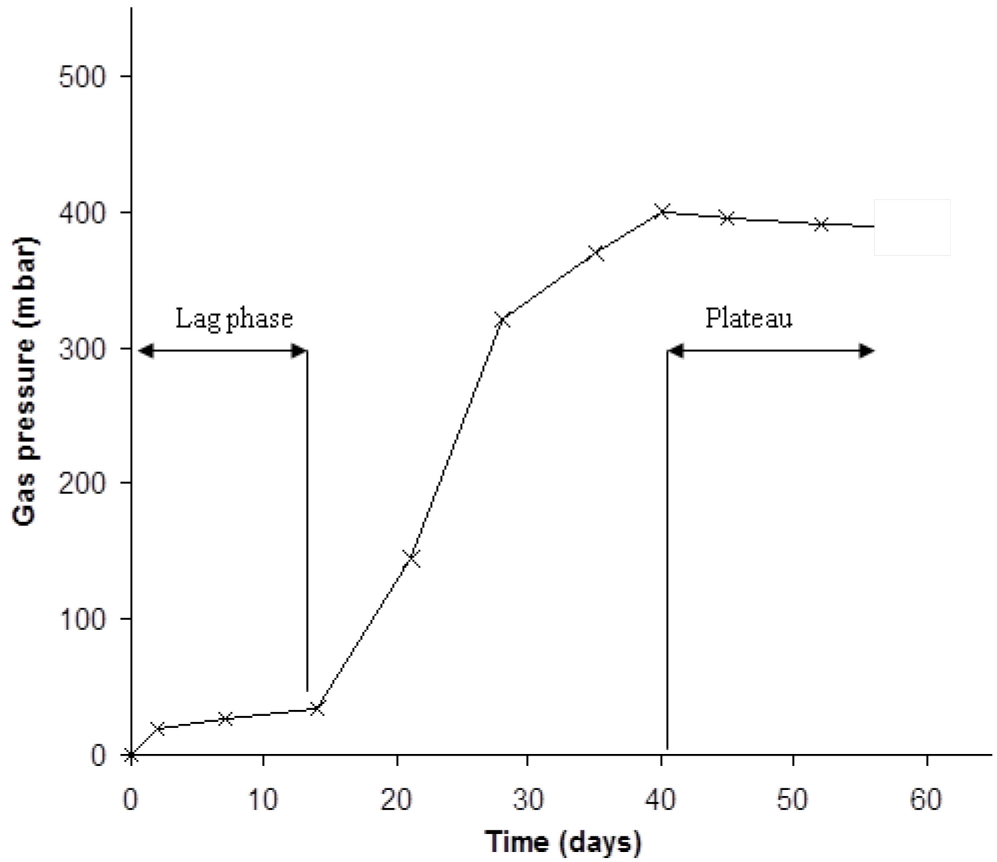
- Inject 1 ml of air at 35°C ± 2°C into each bottle. Insert the needle (on the meter) through the seal of one of the bottles and allow the pressure reading to stabilise. Record the pressure, open the valve until the pressure reads zero and then close the valve;

- Repeat the procedure for the remaining bottles;

- Repeat the total procedure above using 2 ml, 3 ml, 4 ml, 5 ml, 6 ml, 8 ml, 10 ml, 12 ml, 16 ml, 20 ml and 50 ml of air;
-
- Plot a conversion curve of pressure (Pa) against gas volume injected Vb (ml). The response of the instrument is linear over the range 0 Pa to 70 000 Pa, and 0 ml to 50 ml of gas production.

APPENDIX 3

EXAMPLE OF A DEGRADATION CURVE (CUMULATIVE NET PRESSURE INCREASE)



APPENDIX 4

EXAMPLE OF DATA SHEETS FOR THE ANAEROBIC BIODEGRADATION TEST – DATA SHEET FOR THE TEST SUBSTANCE

Laboratory: ..... Test substance: ..... Test No.: .....

Test temperature:(°C): ..... Volume of headspace (V_h): .... (l) Volume of liquid (V_l) : ..... (l)

Carbon in test substance $C_{c,v}$: ..... (mg/l) m_v[1]: ..... (mg)

Day	p_1 (test) (mbar)	p_2 (test) (mbar)	p_3 (test) (mbar)	p (test) mean (mbar)	p_4 (blank) (mbar)	p_5 (blank) (mbar)	p_6 (blank) (mbar)	p (blank) mean (mbar)	p (net) test – blank mean (mbar)	Δp (net) Cumulative (mbar)	m_h headspace C[2] (mg)	D_h Biodegradation[3] (%)
	$C_{IC,1}$ test (mg)	$C_{IC,2}$ test (mg)	$C_{IC,3}$ test (mg)	C_{IC} test mean (mg)	$C_{IC,4}$ blank (mg)	$C_{IC,5}$ blank (mg)	$C_{IC,6}$ blank (mg)	C_{IC} blank mean (mg)	$C_{IC,net}$ test -blank mean (mg)	m_l liquid C[4] (mg)	m_t total C[5] (mg)	D_t Biodegradation[6] (%)
IC (end)												
pH (end)												

[1] Carbon in test vessel, m_v (mg): $m_v = C_{C,v} \times V_l$

[2] Carbon in headspace, m_h (mg) at normal incubation temperature (35 °C): $m_h = 0.468 \Delta p \times V_h$

[3] Biodegradation calculated from headspace gas, D_h (%): $D_h = (m_h \times 100) / m_v$

[4] Carbon in liquid, m_l (mg): $m_l = C_{IC,net} \times V_l$

[5] Total gasified carbon, m_t (mg): $m_t + m_l$

[6] Total biodegradation, D_t (%): $D_t = (m_t \times 100) / m_v$

EXAMPLE OF DATA SHEETS FOR THE ANAEROBIC BIODEGRADATION TEST – DATA SHEET FOR THE REFERENCE SUBSTANCE

Laboratory: ..... Reference substance:..... Test No.:.....
Test temperature:(°C): ..... Volume of headspace (V_h): .....(l) Volume of liquid (V_l) (litres):.....
Carbon in reference substance $C_{c,v}$ (mg/l):..... m_v[1](mg):

Day	p_1 (ref.) (mbar)	p_2 (ref.) (mbar)	p_3 (ref.) (mbar)	p (ref.) mean (mbar)	p_4 (inhib.) (mbar)	p_5 (inhib.) (mbar)	p_6 (inhib.) (mbar)	p (inhib.) mean (mbar)	p (ref.) ref. - blank (mbar)	Δp (ref.) cumulative (mbar)	m_h headspace C^2 (mg)	D_h Biodegradation[3] (%)

[1] Carbon in test vessel, m_v (mg): $m_v = C_{C,v} \times V_l$

[2] Carbon in headspace , m_h (mg) at normal incubation temperature (35 °C): $m_h = 0.468 \Delta p \times V_h$

[3] Biodegradation calculated from headspace gas, D_h (%): $D_h = (m_h \times 100) / m_v$

	$C_{IC,\,1}$ ref. (mg)	$C_{IC,\,2}$ ref. (mg)	$C_{IC,\,3}$ ref. (mg)	C_{IC} ref. mean (mg)	$C_{IC,\,4}$ inhib. (mg)	$C_{IC,\,5}$ inhib. (mg)	$C_{IC,\,6}$ inhib. (mg)	C_{IC} inhib. mean (mg)	$C_{IC,\,net}$ ref. - inhib. (mg)	m_l liquid C[1] (mg)	m_t total C[2] (mg)	D_t Biodegradation[3] (%)
IC (end)												
pH (end)												

[1] Carbon in liquid, m_l (mg): $m_l = C_{IC,net} \times V_l$

[2] Total gasified carbon, m_t (mg): $m_t + m_l$

[3] Total biodegradation, D_t (%): $D_t = (m_t \times 100) / m_v$

C.44. Leaching in Soil Columns

INTRODUCTION

1. This test method is equivalent to OECD Test Guideline (TG) 312 (2004). Man-made chemicals may reach soil directly via deliberate application (e.g. agrochemicals) or via indirect routes (e.g. via waste water → sewage sludge → soil or air → wet/dry deposition). For risk assessment of these chemicals, it is important to estimate their potential for transformation in soil and for movement (leaching) into deeper soil layers and eventually into groundwater.

2. Several methods are available to measure the leaching potential of chemicals in soil under controlled laboratory conditions, i.e. soil thin-layer chromatography, soil thick-layer chromatography, soil column chromatography, and adsorption - desorption measurements (1)(2). For non-ionised chemicals, the n-octanol-water partition coefficient (P_{ow}) allows an early estimation of their adsorption and leaching potential (3)(4)(5).

3. The method described in this test method is based on soil column chromatography in disturbed soil (see Appendix 1 for definition). Two types of experiments are performed to determine (i) the leaching potential of the test chemical, and (ii) the leaching potential of transformation products (study with aged residues) in soils under controlled laboratory conditions[1]. The test method is based on existing methods (6)(7)(8)(9)(10)(11).

4. An OECD Workshop on soil/sediment selection, held at Belgirate, Italy in 1995 (12) agreed on the number and type of soils for use in this test method. It also made recommendations with regard to collection, handling and storage of soil samples for leaching experiments.

PRINCIPLE OF THE TEST METHOD

5. Columns made of suitably inert material (e.g. glass, stainless steel, aluminium, teflon, PVC, etc.) are packed with soil and afterwards saturated and equilibrated with an "artificial rain" solution (for definition see Appendix 1) and allowed to drain. Then the surface of each soil column is treated with the test chemical and/or with aged residues of the test chemical. Artificial rain is then applied to the soil columns and the leachate is collected. After the leaching process the soil is removed from the columns and is sectioned into an appropriate number of segments depending on the information required from the study. Each soil segment and the leachate are then analysed for the

[1] Column leaching studies with crop protection products may provide mobility information on a test chemical and its transformation products and may supplement batch sorption studies.

test chemical and, if appropriate, for transformation products or other chemicals of interest.

APPLICABILITY OF THE TEST METHOD

6. The test method is applicable to test chemicals (unlabelled or radio-labelled: e.g. ^{14}C) for which an analytical method with sufficient accuracy and sensitivity is available. The test method should not be applied to chemicals which are volatile from soil and water and thus do not remain in soil and/or leachate under the experimental conditions of this test method.

INFORMATION ON THE TEST CHEMICAL

7. Unlabelled or radio-labelled test chemicals can be used to measure the leaching behaviour in soil columns. Radio-labelled material is required for studying the leaching of transformation products (aged residues of the test chemical) and for mass balance determinations. ^{14}C-labelling is recommended but other isotopes, such as ^{13}C, ^{15}N, ^{3}H, ^{32}P, may also be useful. As far as possible, the label should be positioned in the most stable part(s) of the molecule. The purity of the test chemical should be at least 95%.

8. Most chemicals should be applied as single substance However, for active substances in plant protection products, formulated products may be used to study the leaching of the parent test substance but their testing is particularly required when the mixture is likely to affect the release rate (e.g. granular or controlled release formulations). Regarding mixture specific requirements for test design, it may be useful to consult with the regulatory authority prior to conducting the test. For aged residue leaching studies, the pure parent test substance should be used.

9. Before carrying out leaching tests in soil columns, the following information on the test chemical should preferably be available:

   (1) solubility in water [test method A.6] (13);

   (2) solubility in organic solvents;

   (3) vapour pressure [test method A.4] (13) and Henry's Law constant;

   (4) n-octanol/water partition coefficient [test methods A.8 and A.24] (13);

   (5) adsorption coefficient (K_d, K_f or K_{OC}) [test methods C.18 and/or C.19] (13);

   (6) hydrolysis [test method C.7] (13);

   (7) dissociation constant (pK_a) [OECD TG 112] (25);

   (8) aerobic and anaerobic transformation in soil [test method C.23] (13)

Note: The temperature at which these measurements were made should be reported in

the respective test reports.

10. The amount of test chemical applied to the soil columns should be sufficient to allow for detection of at least 0.5 % of the applied dose in any single segment. For active chemicals in plant protection products, the amount of test chemical applied may correspond to the maximum recommended use rate (single application).

11. An appropriate analytical method of known accuracy, precision and sensitivity for the quantification of the test chemical and, if relevant, of its transformation products in soil and leachate must be available. The analytical detection limit for the test chemical and its significant transformation products (normally at least all transformation products ≥10 % of applied dose observed in transformation pathway studies, but preferably any relevant transformation products of concern) should also be known (see paragraph 17).

REFERENCE CHEMICALS

12. Reference chemicals with known leaching behaviour such as atrazine or monuron which can be considered moderate leachers in the field should be used for evaluating the relative mobility of the test chemical in soil (1)(8)(11). A nonsorbing and non degradable polar reference chemical (e.g. tritium, bromide, fluorescein, eosin) to trace the movement of water in the column may also be useful to confirm the hydrodynamic properties of the soil column.

13. Analytical standard chemicals may also be useful for the characterisation and/or identification of transformation products found in the soil segments and in the leachates by chromatographic, spectroscopic or other relevant methods.

DEFINITIONS AND UNITS

14. See Appendix 1.

QUALITY CRITERIA

Recovery

15. The sum of the percentages of the test chemical found in the soil segments and the column leachate after leaching gives the recovery for a leaching experiment. Recoveries should range from 90 % to 110 % for radio-labelled chemicals (11) and from 70 % to 110 % for non-labelled chemicals (8).

Repeatability and sensitivity of analytical method

16. Repeatability of the analytical method to quantify test chemical and transformation products can be checked by duplicate analysis of the same extract of a soil segment or

of a leachate (see paragraph 11).

17. The limit of detection (LOD) of the analytical method for the test chemical and for the transformation products should be at least 0.01 mg • kg^{-1} in each soil segment or leachate (as test chemical) or 0.5 % of applied dose in any single segment whichever is lower. The limit of quantification (LOQ) should also be specified.

DESCRIPTION OF THE TEST METHOD

Test system

18. Leaching columns (sectionable or non-sectionable) made of suitably inert material (e.g. glass, stainless steel, aluminium, teflon, PVC, etc.) with an inner diameter of at least 4 cm and a minimum height of 35 cm are used for the test. Column materials should be tested for potential interactions with the test chemical and/or its transformation products. Examples of suitable sectionable and non-sectionable columns are shown in Appendix 2.

19. Spoon, plunger and vibration apparatus are used for filling and packing the soil columns.

20. For application of artificial rain to the soil columns, piston or peristaltic pumps, showering heads, Mariotte bottles or simple dropping funnels can be used.

Laboratory equipment and chemicals

21. Standard laboratory equipment is required, in particular the following:

    (1) analytical instruments such as GLC, HPLC and TLC equipment, including the appropriate detection systems for analysing labelled or unlabelled chemicals or inverse isotope dilution method;

    (2) instruments for identification purposes (e.g. MS, GC-MS, HPLC-MS, NMR, etc.);

    (3) liquid scintillation counter for radio-labelled test chemical;

    (4) oxidiser for combustion of labelled material;

    (5) extraction apparatus (for example, centrifuge tubes for cold extraction and Soxhlet apparatus for continuous extraction under reflux);

    (6) instrumentation for concentrating solutions and extracts (e.g. rotating evaporator).

22. Chemicals used include: organic solvents, analytical grade, such as acetone, methanol, etc.; scintillation liquid; 0.01 M $CaCl_2$ solution in distilled or deionised water (= artificial rain).

Test chemical

23. To apply the test chemical to the soil column it should be dissolved in water (deionised or distilled). If the test chemical is poorly soluble in water, it can be applied either as formulated product (if necessary after suspending or emulsifying in water) or in any organic solvent. In case an organic solvent is used, it should be kept to a minimum and should be evaporated from the surface of the soil column prior to start of leaching procedure. Solid formulations, such as granules, should be applied in the solid form without water; to allow a better distribution over the surface of the soil column, the formulated product may be mixed with a small amount of quartz sand (e.g. 1 g) before application.

24. The amount of test chemical applied to the soil columns should be sufficient to allow for detection of at least 0.5 % of the applied dose in any single segment. For active chemicals in plant protection products, this may be based on the maximum recommended use rate (single application rate) and, for both parent and aged leaching, should be related to the surface area of the soil column used[1].

Reference chemical

25. A reference chemical should be used in the leaching experiments (see paragraph 12). It should be applied to the soil column surface in a similar way as the test chemical and at an appropriate rate that enables adequate detection either as an internal standard together with the test chemical on the same soil column or alone on a separate soil column. It is preferred that both chemicals be run on the same column, except when both chemicals are similarly labelled.

Soils

Soil selection

26. For leaching studies with the parent test chemical 3 to 4 soils with varying pH, organic carbon content and texture should be used (12). Guidance for selection of soils for leaching experiments is given in Table 1 below. For ionisable test chemicals the

---

[1] The amount to be applied to cylindrical soil columns can be calculated by the following formula:

$$M\,[\mu g] = \frac{A\,[kg/ha] \bullet 10^9\,[\mu g/kg] \bullet d^2\,[cm^2] \bullet \pi}{10^8\,[cm^2/ha] \bullet 4}$$

where:
M = amount applied per column [µg]
A = rate of application [kg · ha^{-1}]
d = diameter of soil column [cm]
π = 3.14

selected soils should cover a wide range of pH, in order to evaluate the mobility of the chemical in its ionised and unionised forms; at least 3 soils should have a pH at which the test chemical is in its mobile form.

Table 1: Guidance for selection of soils for leaching studies

Soil No.	pH value	Organic carbon %	Clay content %	Texture*
1	> 7.5	3.5 - 5.0	20 - 40	clay loam
2	5.5 - 7.0	1.5 - 3.0	15 - 25	silt loam
3	4.0 - 5.5	3.0 - 4.0	15 - 30	loam
4	<4.0 - 6.0 §	<0.5 - 1.5 § ‡	<10 - 15 §	loamy sand
5	< 4.5	> 10#	< 10	loamy sand/sand

* According to FAO and USDA systems (14).

§ The respective variables should preferably show values within the range given. If, however, difficulties in finding appropriate soil material occur, values below the indicated minimum are accepted.

‡ Soils with less than 0.3 % organic carbon may disturb correlation between organic content and adsorption. Thus, it is recommended to use soils with a minimum organic carbon content of 0.3 %.

Soils with very high carbon content (e.g. >10%) may not be acceptable legally e.g. for pesticide registration purposes.

27. Other soil types may sometimes be necessary to represent cooler, temperate and tropical regions. Therefore, if other soil types are preferred, they should be characterised by the same parameters and should have similar variations in properties as those described in the guidance for selection of soils for leaching studies (see Table 1 above), even if they do not match the criteria exactly.

28. For leaching studies with "aged residues", one soil should be used (12). It should have a sand content > 70% and an organic carbon content between 0.5 - 1.5 % (e.g. soil No. 4 in Table 1). Use of more soil types may be necessary if data on the transformation products are important.

29. All soils should be characterised at least for texture [% sand, % silt, % clay according to FAO and USDA classification systems (14)], pH, cation exchange capacity, organic carbon content, bulk density (for disturbed soil) and water holding capacity. Measurement of microbial biomass is only required for the soil which is used in the ageing/incubation period carried out before the aged leaching experiment. Information on additional soil properties (e.g. soil classification, clay mineralogy, specific surface area) may be helpful for interpreting the results of this study. For determination of soil characteristics the methods recommended in references (15)(16)(17)(18)(19) can be used.

Collection and storage of soils

30. The soils should be taken from the top layer (A-horizon) to a maximum depth of 20 cm. Remains of vegetation, macro-fauna and stones should be removed. The soils

(except those used for ageing the test chemical) are air-dried at room temperature (preferably between 20-25 C). Disaggregation should be performed with minimal force, so that the original texture of the soil will be changed as little as possible. The soils are sieved through a ≤ 2 mm sieve. Careful homogenisation is recommended, as this enhances the reproducibility of the results. Before use the soils can be stored at ambient temperature and kept air dried (12). No limit on storage time is recommended but soils stored for more than 3 years should be re-analysed prior to use with respect to their organic carbon content and pH.

31. Detailed information on the history of the field sites from where the test soils are collected should be available. Details include exact location [exactly defined by UTM (Universal Transversal Mercator-Projection/European Horizontal Datum) or geographical co-ordinates], vegetation cover, treatments with crop protection chemicals, treatments with organic and inorganic fertilisers, additions of biological materials or accidental contamination (12). If soil has been treated with the test chemical or its structural analogues within the previous four years, these soils should not be used for leaching studies.

Test conditions

32. During the test period, the soil leaching columns should be kept in the dark at ambient temperature as long as this temperature is maintained within a range of ±2°C. Recommended temperatures are between 18 and 25°C.

33. Artificial rain (0.01 M $CaCl_2$) should be applied continuously to the surface of the soil columns at a rate of 200 mm over a period of 48 hours[1]; this rate is equivalent to an application of 251 ml for a column with an inner diameter of 4 cm. If needed for the purpose of the test, other rates of artificial rainfall and longer duration may additionally be used.

Performance of the test

Leaching with parent test chemical

34. At least duplicate leaching columns are packed with untreated, air-dried and sieved soil (< 2 mm) up to a height of approximately 30 cm. To obtain uniform packing, the soil is added to the columns in small portions with a spoon and pressed with a plunger under simultaneous gentle column vibration until the top of the soil column does not sink in further. Uniform packing is required for obtaining reproducible results from leaching columns. For details on column packing techniques, see references (20)(21) and (22). To control the reproducibility of the packing procedure, the total weight of the soil packed in the columns is determined[2]; the weights of the duplicate columns

[1] This simulates an extremely high rainfall. The average yearly rainfall, for example, in Central Europe is of the order of 800-1000mm.

[2] Examples of bulk densities for disturbed soils are as follows:

should be similar.

35. After packing, the soil columns are pre-wetted with artificial rain (0.01 M $CaCl_2$) from bottom to top in order to displace the air in the soil pores by water. Thereafter the soil columns are allowed to equilibrate and the excess water is drained off by gravity. Methods for column saturation are reviewed in reference (23).

36. Then the test chemical and/or the reference chemical are applied to the soil columns (see also paragraphs 23-25). To obtain a homogeneous distribution the solutions, suspensions or emulsions of the test and/or reference chemical should be applied evenly over the surface of the soil columns. If incorporation into soil is recommended for the application of a test chemical, it should be mixed in a small amount (e.g. 20 g) of soil and added to the surface of the soil column.

37. The surfaces of the soil columns are then covered by a glass sinter disk, glass pearls, glass fibre filters or a round filter paper to distribute the artificial rain evenly over the entire surface and to avoid disturbance of the soil surface by the rain drops. The larger the column diameter the more care is needed for the application of the artificial rain to the soil columns to ensure an even distribution of the artificial rain over the soil surface. Then the artificial rainfall is added to the soil columns drop-wise with the aid of a piston or a peristaltic pump or a dropping funnel. Preferably, the leachates should be collected in fractions and their respective volumes are recorded[1].

38. After leaching and allowing the columns to drain, the soil columns are sectioned in an appropriate number of segments depending on the information required from the study, the segments are extracted with appropriate solvents or solvent mixtures and analysed for the test chemical and, when appropriate, for transformation products, for total radioactivity and for the reference chemical. The leachates or leachate fractions are analysed directly or after extraction for the same products. When radio-labelled test chemical is used, all fractions containing ≥ 10 % of the applied radioactivity should be identified.

Leaching with aged residues

39. Fresh soil (not previously air-dried) is treated at a rate corresponding to the surface area of the soil columns (see paragraph 24) with the radio-labelled test chemical and incubated under aerobic conditions according to Test Method C.23 (13). The incubation (ageing) period should be long enough to produce significant amounts of transformation products; an ageing period of one half-life of the test chemical is

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for a sand soil 1.66 g • ml^{-1} for a loamy sand soil 1.58 g • ml^{-1}
for a loam soil 1.17 g • ml^{-1} for a silt soil 1.11 • g ml^{-1}

[1] Typical leachate volumes range from 230-260 ml corresponding to approx. 92-104 % of total artificial rain applied (251 ml) when using soil columns of 4 cm diameter and 30 cm length.

recommended[1], but should not exceed 120 days. Prior to leaching, the aged soil is analysed for the test chemical and its transformation products.

40. The leaching columns are packed up to a height of 28 cm with the same soil (but air-dried) as used in the ageing experiment as described in paragraph 34 and the total weight of the packed soil columns is also determined. The soil columns are then pre-wetted as described in paragraph 35.

41. Then the test chemical and its transformation products are applied to the surface of the soil columns in the form of aged soil residues (see paragraph 39) as a 2 cm soil segment. The total height of the soil columns (untreated soil + aged soil) should preferably not exceed 30 cm (see paragraph 34).

42. The leaching is carried out as described in paragraph 37.

43. After leaching, soil segments and leachates are analysed as indicated in paragraph 38 for the test chemical, its transformation products and not-extracted radioactivity. To determine how much of the aged residue is retained in the top 2-cm layer after leaching, this segment should be analysed separately.

DATA AND REPORTING

Treatment of results

44. The amounts of test chemical, transformation products, non-extractables and, if included, of the reference chemical should be given in % of applied initial dose for each soil segment and leachate fraction. A graphical presentation should be given for each column plotting the percentages found as a function of the soil depths.

45. When a reference chemical is included in these column leaching studies, the leaching of a chemical can be evaluated on a relative scale using relative mobility factors (RMF; for definition see Appendix 3) (1)(11) which allows the comparison of leaching data of various chemicals obtained with different soil types. Examples of RMF-values for a variety of crop protection chemicals are given in Appendix 3.

46. Estimates of K_{oc} (organic carbon normalised adsorption coefficient) and K_{om} (organic matter normalised distribution coefficient) can also be obtained from column leaching results by using average leaching distance or established correlations between RMF and K_{om} respectively K_{oc} (4) or by applying simple chromatographic theory (24). However, the latter method should be used with caution especially when considering that the leaching process does not solely involve saturated flow conditions, but rather

[1] More than one major transformation product may be formed in soil which also may appear at different time points during a transformation study. In such cases, it may be necessary to conduct leaching studies with aged residues of different age.

unsaturated systems.

Interpretation of results

47. The column leaching studies described in this method allow determining the leaching or mobility potential in soil of the test chemical (in the parent leaching study) and/or its transformation products (in the aged residue leaching study). These tests do not quantitatively predict leaching behaviour under field conditions, but they can be used to compare the 'leachability' of one chemical with others whose leaching behaviour may be known (24). Likewise, they do not quantitatively measure the percentage of applied chemical that might reach ground water (11). However, the results of column leaching studies may assist in deciding whether additional semi-field or field testing has to be carried out for chemicals showing a high mobility potential in laboratory tests.

Test report

48. The report must include:

Test chemical and reference chemical (when used):

- common name, chemical name (IUPAC and CAS nomenclature), CAS number, chemical structure (indicating position of label when radio-labelled material is used) and relevant physical-chemical properties;
- purities (impurities) of test chemical;
- radiochemical purity of labelled chemical and specific activity (where appropriate).

Test soils:

- details of collection site;
- properties of soils, such as pH, organic carbon and clay content, texture and bulk density (for disturbed soil);
- soil microbial activity (only for soil used for ageing of test chemical);
- length of soil storage and storage conditions.

Test conditions:

- dates of the performance of the studies;
- length and diameter of leaching columns;
- total soil weight of soil columns;
- amount of test chemical and, if appropriate, reference chemical applied;
- amount, frequency and duration of application of artificial rain;
- temperature of experimental set-up;
- number of replications (at least two);
- methods for analysis of test chemical, transformation products and, where appropriate, of reference chemical in the various soil segments and leachates;
- methods for the characterisation and identification of transformation products in the soil segments and leachates.

Test results:

- tables of results expressed as concentrations and as % of applied dose for soil segments and leachates;

- mass balance, if appropriate;
- leachate volumes;
- leaching distances and, where appropriate, relative mobility factors;
- graphical plot of % found in the soil segments versus depth of soil segment;
- discussion and interpretation of results.

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Chapter A.4, vapour pressure
Chapter A.6, Water solubility
Chapter A.8, Partition coefficient, shake flask method
Chapter A.24, Partition coefficient, HPLC method
Chapter C.7, degradation — abiotic degradation: hydrolysis as a function of pH
Chapter C.18, Adsorption/desorption using a batch equilibrium method
Chapter C.23, Aerobic and anaerobic transformation in soil

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APPENDIX 1

DEFINITIONS AND UNITS

Aged soil residue: Test chemical and transformation products present in soil after application and following a period long enough to allow transport, adsorption, metabolism, and dissipation processes to alter the distribution and chemical nature of some of the applied chemical (1).

Artificial rain: 0.01 M $CaCl_2$ solution in distilled or deionised water.

Average Leaching Distance: Bottom of soil section where cumulative recovered chemical = 50% of total recovered test chemical [normal leaching experiment], or; (bottom of soil section where cumulative recovered chemical = 50% of total recovered test chemical) – ((thickness of aged residue layer)/2) [aged residue leaching study]

Chemical: a substance or a mixture.

Leachate: Aqueous phase percolated through a soil profile or a soil column (1).

Leaching: Process by which a chemical moves downward through the soil profile or a soil column (1).

Leaching distance: Deepest soil segment in which ≥ 0.5 % of the applied test chemical or aged residue was found after the leaching process (equivalent to penetration depth).

Limit of detection (LOD) and **limit of quantification (LOQ)**: The limit of detection (LOD) is the concentration of a chemical below which the identity of the chemical cannot be distinguished from analytical artefacts. The limit of quantification (LOQ) is the concentration of a chemical below which the concentration cannot be determined with an acceptable accuracy.

RMF Relative Mobility Factor: (leaching distance of test chemical (cm)) / (leaching distance of reference chemical (cm))

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

Transformation product: All chemicals resulting from biotic or abiotic transformation reactions of the test chemical including CO_2 and products that are bound in residues.

Soil: A mixture of mineral and organic chemical constituents, the latter containing compounds of high carbon and nitrogen content and of high molecular weights, populated by small (mostly micro-) organisms. Soil may be handled in two states:

- undisturbed, as it has developed with time, in characteristic layers of a variety of soil types;
- disturbed, as it is usually found in arable fields or as occurs when samples are taken by digging and used in this test method (2).

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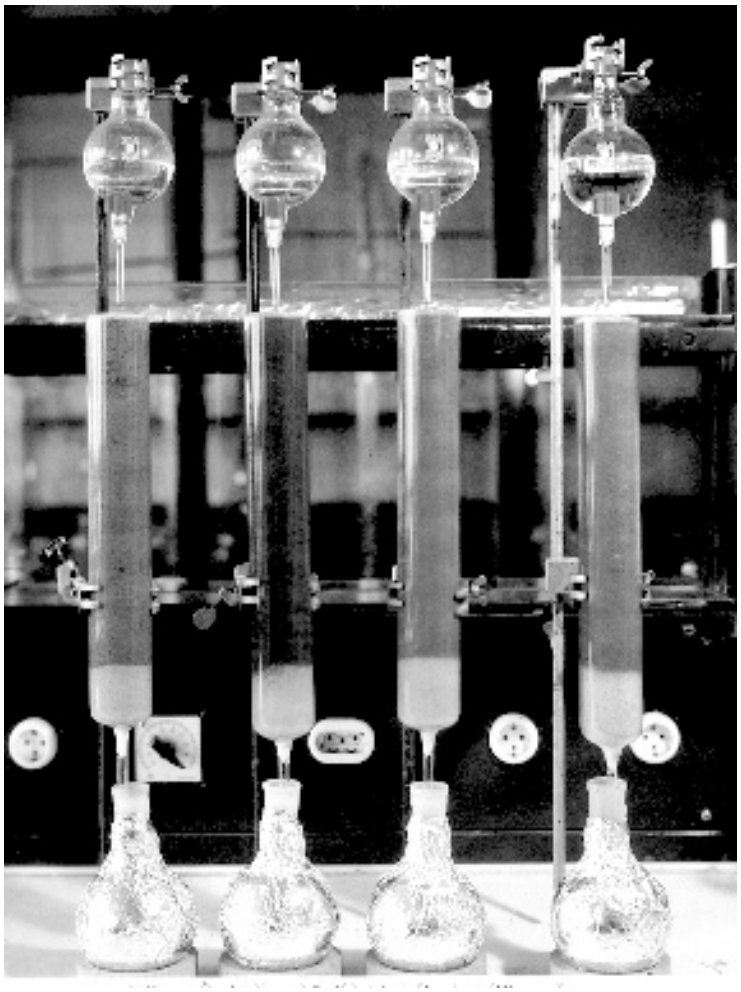
APPENDIX 2

Figure 1: Example of non- sectionable leaching columns made of glass With a length of 35 cm and an inner diameter of 5 cm (1)



← Dropping funnels for application of artificial rain

← Glass sinter disc to avoid disturbance of the soil surface and to evenly distribute the artificial rain

← Glass column filled with test soil (when testing photolabile products columns should be wrapped in aluminium foil)

← Layer of quartz sand

← Glass wool plug to keep the soil in the column

← Round-bottom flask for collection of leachate; wrapped in aluminium foil to exclude photolysis

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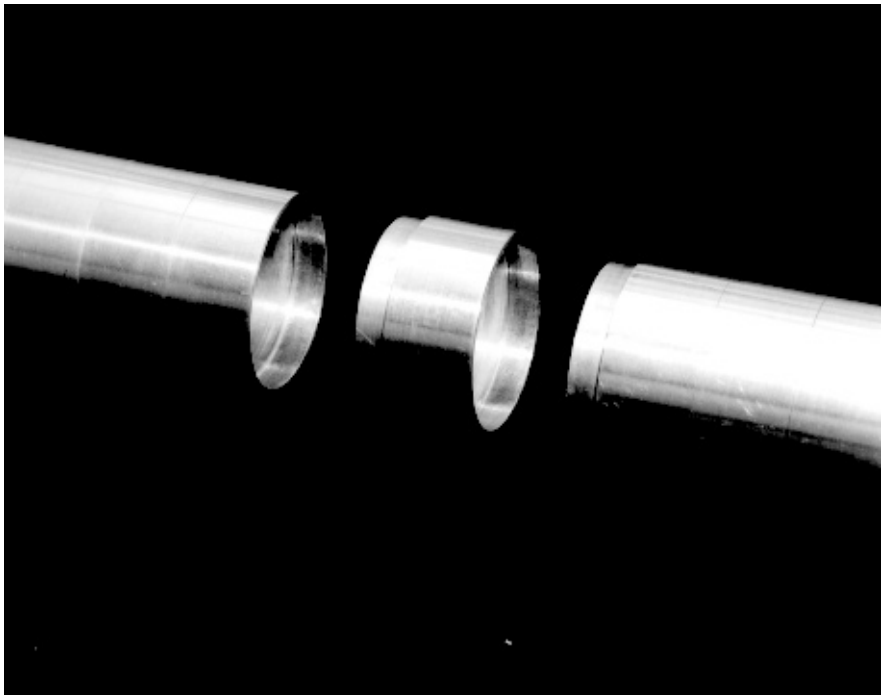
Figure 2: Example of a sectionable metal column with 4 cm inner diameter (1)



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APPENDIX 3

Examples of Relative Mobility Factors* (RMF) for a variety of Crop protection chemicals (1)(2) and corresponding mobility classes+

RMF-Range	Chemical (RMF)	Mobility Class
≤ 0.15	Parathion (< 0.15), Flurodifen (0.15)	I immobile
0.15 - 0.8	Profenophos (0.18), Propiconazole (0.23), Diazinon (0.28), Diuron (0.38), Terbuthylazine (0.52), Methidathion (0.56), Prometryn (0.59), Propazine (0.64), Alachlor (0.66), Metolachlor (0.68)	II slightly mobile
0.8 - 1.3	Monuron** (1.00), Atrazine (1.03), Simazine (1.04), Fluometuron (1.18)	III moderately mobile
1.3 - 2.5	Prometon (1.67), Cyanazine (1.85), Bromacil (1.91), Karbutilate (1.98)	IV fairly mobile
2.5 - 5.0	Carbofuran (3.00), Dioxacarb (4.33)	V mobile
> 5.0	Monocrotophos (> 5.0), Dicrotophos (> 5.0)	VI very mobile

* The Relative Mobility Factor is derived as follows (3):

$$RMF = \frac{\text{leaching distance of test chemical (cm)}}{\text{leaching distance of reference chemical (cm)}}$$

** Reference chemical

+ Other systems to classify a chemical's mobility in soil are based on R_f values from soil thin-layer chromatography (4) and on K_{oc} values (5)(6).

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C.45. Estimation of Emissions from Preservative - Treated Wood to the Environment: Laboratory Method for Wooden Commodities that are not Covered and are in Contact with Fresh Water or Seawater

INTRODUCTION

1. This test method is equivalent to OECD test guideline (TG) 313 (2007). The emissions from preservative-treated wood to the environment need to be quantified to enable an environmental risk assessment of the treated wood. This test method describes a laboratory method for the estimation of emissions from preservative-treated wood in two situations where emissions could enter the environment:

- Emissions from treated wood in contact with fresh water. Emissions from the surface of the treated wood could enter the water.
- Emissions from treated wood in contact with seawater. Emissions from the surface of the treated wood could enter the seawater.

2. This test method is intended for testing the emissions from wood and wooden commodities that are not covered and are in contact with fresh water or seawater. Use Classes are used internationally and categorise the biological hazard to which the treated commodity will be subjected. Use Classes also define the situation in which the treated commodity is used and determine the environmental compartments (air, water, soil) which are potentially at risk from the preservative treated wood.

3. The test method is a laboratory procedure for obtaining samples (emissate) from water used to immerse treated wood, at increasing time intervals after exposure. The quantity of emissions in the emissate is related to the surface area of the wood and the length of exposure, to estimate a flux in $mg/m^2/day$. The flux (leaching rate) after increasing periods of exposure can thus be estimated.

4. The quantity of emissions can be used in an environmental risk assessment of the treated wood.

INITIAL CONSIDERATIONS

5. The mechanism of leaching at the wood surface by fresh water is not assumed to be identical in nature and severity to leaching from a wood surface by seawater. Thus, for wood preservative products or mixtures used to treat wood used in seawater environs, a wood leaching study for seawater is necessary.

6. The wood, in the case of wood treated with a wood preservative, should be representative of commercially used wood. It should be treated in accordance with the preservative manufacturer's instructions and in compliance with appropriate standards and specifications. The parameters for the post treatment conditioning of the wood prior to the commencement of the test should be stated.

7. The wood samples used should be representative of the commodities used (e.g., with regard to species, density and other characteristics).

8. The test can be applied to wood using a penetrating process or superficial application or to treated wood which has an additional mandatory surface treatment (e.g., paint that is applied as a requirement for commercial use).

9. The composition, amount, pH and the physical form of water is important in determining the quantity, content and nature of emissions from wood.

PRINCIPLE OF THE TEST METHOD

10. Preservative-treated wood test specimens are immersed in water. The water (emissate) is collected and chemically analysed multiple times over the exposure period sufficient to perform statistical calculations. Emission rates in $mg/m^2/day$ are calculated from analytical results. The sampling periods should be recorded. Tests with untreated samples can be discontinued if there is no background detected in the first three data points.

11. The inclusion of untreated wood specimens allows for the determination of background levels for emissates from wood other than the preservative used.

QUALITY CRITERIA

Accuracy

12. The accuracy of the test method to estimate emission depends upon the test specimens being representative of commercially treated wood, how representative the water is of real water and how the exposure regime is representative of natural conditions.

13. The accuracy, precision and repeatability of the analytical method should be determined before conducting the test.

Reproducibility

14. Three water samples are collected and analysed and the mean value is taken as the emission value. The reproducibility of the results within one laboratory and between different laboratories depends upon the immersion regime and the wood used as test specimens.

Acceptable Range of Results

15. A range of results from this test where the upper and lower values differ by less than one order of magnitude is acceptable.

TEST CONDITIONS

Water

16. Freshwater leaching scenarios: Deionised water (e.g., ASTM D 1193 Type II) is recommended for use in the leaching test when wood exposed to freshwater is to be evaluated. The water temperature shall be 20 °C +/- 2 °C and the measured pH and water temperature included in the test report. Analysis of samples of the water used taken before immersion of the treated specimens allows the estimation of the analysed chemicals in the water. This is a control to determine background levels of chemicals which are then chemically analysed.

17. Seawater leaching scenarios: Synthetic seawater (e.g., ASTM D 1141 Substitute Ocean Water, without Heavy Metals) is recommended for use in the leaching test when wood exposed to seawater is to be evaluated. The water temperature shall be 20 °C +/- 2 °C and the measured pH and water temperature included in the test report. Analysis of samples of the water used taken before immersion of the treated specimens allows the estimation of the analysed chemicals in the water. This is a control for the analysis of background levels for chemicals of importance.

Wood Test Specimens

18. The wood species should be typical of the wood species used for the efficacy testing of wood preservatives. The recommended species are *Pinus sylvestris L.* (Scots pine), *Pinus resinosa Ait*. (red pine) or *Pinus spp* (Southern pine). Additional tests may be made using other species.

19. Straight grained wood without knots should be used. Material of a resinous appearance should be avoided. The wood should be typical of wood which is available commercially. The source, density and number of annual rings per 10 mm should be recorded.

20. Wood test specimens are recommended to be sets of five according to EN 113 size blocks (25 mm x 50 mm x 15 mm dimensions) with the longitudinal faces parallel to the grain of the wood, although other dimensions such as 50 mm, by 150 mm, by 10 mm may be used. The test specimen should be completely immersed into the water. Test specimens shall consist of 100% sapwood. Each specimen is uniquely marked so that it can be identified throughout the test.

21. All test specimens should be planed or plane sawn and the surfaces should not be sanded.

22. The number of sets of wood test specimens used for analysing is at least five: three sets of specimens are treated with preservative, one set of specimens is untreated and one set of specimens for the estimation of the oven dry moisture content of the test specimens before treatment. Sufficient test specimens are prepared to allow selection of three sets of specimens which are within 5% of the mean value of the preservative retentions of the pool of test specimens.

23. All test specimens are end-sealed with a chemical which prevents penetration of preservative into the end grain of the specimens or prevents leaching from the specimens via the end grain. It is necessary to distinguish between specimens used for superficial application and penetration processes for the application of the end-sealant. The application of the end-sealant has to be applied prior to treatment only in case of superficial application.

24. The end-grain has to be open for treatments by penetration processes. Therefore, the specimens have to be end-sealed at the end of the conditioning period. The emission has to be estimated for the longitudinal surface area only. Sealants should be inspected and reapplied if necessary prior to initiating leaching and should not be reapplied after leaching has been initiated.

Immersion Container

25. The container is made of an inert material and is large enough to contain 5 EN113 wood specimens in 500 ml of water resulting in a surface area to water volume ratio of 0.4 cm^2/ml.

Specimen Test Assembly

26. The test specimens are supported on an assembly which allows all exposed surfaces of the specimen to be in contact with water.

PROCEDURE FOR PRESERVATIVE TREATMENT

Preparation of the Treated Test Specimens

27. The wood test specimen to be treated with the preservative under test is treated by the method specified for the preservative, which may be by a penetrating treatment process or a superficial application process, which may be with a dip, spray or brush.

Preservatives to be applied by penetrating treatment process

28. A solution of the preservative should be prepared that will achieve the specified uptake or retention when applied using the penetrating treatment process. The wood test specimen is weighed and its dimensions are measured. The penetrating treatment process should be as specified for the application of the preservative to wood for use in Use Class 4 or 5. The specimen is again weighed after treatment and the retention of the preservative (kg/m^3) is calculated from the equation:

$$\frac{\text{Mass after treatment (kg)} - \text{Mass before treatment (kg)}}{\text{Test specimen volume } (m^3)} \times \frac{\text{Solution Concentration (\% mass / mass)}}{100}$$

29. Note that timber treated in an industrial treatment plant (e.g. by vacuum pressure impregnation) may be used in this test. The procedures used should be recorded and the retention of material treated in this way must be analysed and recorded.

Preservatives to be applied by superficial application processes

30. The superficial application process includes dipping, spraying or brushing of the wood test specimens. The process and application rate (e.g. litres/m^2) should be as specified for the superficial application of the preservative.

31. Also note in this case, timber treated in an industrial treatment plant may be used in this test. The procedures used should be recorded and the retention of material treated in this way must be analysed and recorded.

Conditioning of the Test Specimens after Treatment

32. After treatment, the treated test specimens should be conditioned in accordance with the recommendations made by the supplier of the test preservative according to the preservative label requirements or as in accordance with commercial treatment practices or in accordance with EN 252 Standard.

Preparation and Selection of Test Specimens

33. After post treatment conditioning, the mean retention of the group of test specimens is calculated and three representative sets of specimens with a retention within 5% of the mean for the group are randomly selected for leaching measurements.

PROCEDURE FOR PRESERVATIVE EMISSION MEASUREMENTS

Immersion Method

34. The test specimens are weighed and subsequently totally immersed in the water and the date and time recorded. The container is covered to reduce evaporation.

35. The water is replaced at the following intervals: 6 hours, 1 day, 2 days, 4 days, 8 days, 15 days, 22 days, 29 days (note: these are total times not interval times). The time and date of the water change and the mass of water recovered from the container should be recorded.

36. After each water exchange, a sample of water in which the set of test specimens has been immersed is retained for subsequent chemical analysis.

37. The sampling procedure allows the calculation of the profile of the quantity of emissions against time. Samples should be stored under conditions that preserve the analyte e.g., in a refrigerator in the dark to reduce microbial growth in the sample before analysis.

EMISSION MEASUREMENTS

Treated Samples

38. Collected water is chemically analysed for the active substance and/or relevant degradation/transformation products, if appropriate.

Untreated Samples

39. Collection of the water (emissate) in this system and subsequent analysis of chemicals that had leached from the untreated wood samples allow the estimation of the possible emission rate of the preservative from untreated wood. Collection and analysis of the emissate after increasing time periods of exposure allow the rate of change of the emission rate with time to be estimated. This analysis is a control procedure to determine background levels of the test chemical in untreated wood to confirm that the wood used as a source of samples had not been previously treated with the preservative.

DATA AND REPORTING

Chemical Analyses

40. The collected water is chemically analysed and the water analysis result is expressed in appropriate units, e.g., µg/l.

Reporting of Data

41. All results are recorded. The Appendix shows an example of a suggested recording form for one set of treated test specimens, and the summary table for calculating the mean emission values over each sampling interval.

42. The daily emission flux in $mg/m^2/day$ is calculated by taking the mean of the three measurements from the three replicates and dividing by the number of days of immersion.

Test Report

43. At least the following information shall be provided in the test report:

- The name of the supplier of the preservative under test;
- The specific and unique name or code of the preservative tested;
- The trade or common name of the active ingredient(s) with a generic description of the co-formulants (e.g. co-solvent, resin), and the composition in % m/m of the ingredients;
- The relevant retention or loading (in kg/m^3 or l/m^2, respectively) specified for wood used in contact with water;
- The species of wood used, with its density, and growth rate in rings per 10 mm;
- The loading or retention of the preservative tested and the formula used to calculate the retention, expressed as l/m^2 or kg/m^3 ;
- The method of application of the preservative, specifying the treatment schedule used

for a penetrating process, and the method of application if a superficial treatment was used;
- The date of application of the preservative, and an estimate of the moisture content of the test specimens, expressed as a percentage;
- Conditioning procedures used, specifying the type, conditions and duration;
- Specification of the end sealant used and the number of times applied;
- Specification of any subsequent treatment of the wood, e.g. specification of the supplier, type, characteristics and loading of a paint;
- The time and date of each immersion event, the amount of water used for the immersion of the test specimens at each event, and the amount of water absorbed by the wood during immersion;
- Any variation from the described method and any factors that may have influenced the results.

LITERATURE

(1) European Standard, EN 84 – 1997. Wood preservatives. Accelerated ageing of treated wood prior to biological testing. Leaching procedure.

(2) European Standard, EN 113/A1 – 2004. Wood preservatives. Test method for determining the protective effectiveness against wood destroying basidiomycetes. Determination of the toxic values.

(3) European Standard, EN 252 – 1989. Field test method for testing the relative protective effectiveness of a wood preservative in ground contact.

(4) European Standard, EN 335 – Part 1: 2006. Durability of wood and wood-based products – Definition of use classes – Part1: General.

(5) American Society for Testing and Materials Standards, ASTM D 1141 – 1998. Standard Practice for the Preparation of Substitute Ocean Water, Without Heavy Metals. *Annual Book of ASTM Standards*, Volume 11.02.

(6) American Society for Testing and Materials Standards, ASTM D 1193-77 Type II – 1983. Specifications for Reagent Water. *Annual Book of ASTM Standards*, Volume 11.01.

Appendix 1

RECORDING FORM FOR TEST METHOD

Estimation of Emissions from Preservative-Treated Wood to the Environment: Laboratory Method for Wooden Commodities that are not Covered and are in Contact with Fresh Water or Seawater

Test house	
Wood preservative	
Supplier of the preservative	
Specific and unique name or code of the preservative	
Trade or common name of the preservative	
Co-formulants	
Relevant retention for wood used in contact with water	
Application	
Application method	
Date of application	
Formula used to calculate the retention:	
Conditioning procedure	
Duration of conditioning	
End sealant / number of times applied	
Subsequent treatment	if relevant
Test specimens	
Wood species	
Density of the wood	(minimum ... mean value ... maximum)
Growth rate (rings per 10 mm)	(minimum ... mean value ... maximum)
Moisture content	
Test assemblies*	**Retention (e.g. kg/m³)**
Treated ‚x'	Mean value and standard deviation or range for 5 specimens
Treated ‚y'	Mean value and standard deviation or range for 5 specimens
Treated ‚z'	Mean value and standard deviation or range for 5 specimens
Untreated	
Variation of test method parameters	e.g. water quality, dimension of test specimens etc.

* x, y, z represent the three replicate samples

Time	Water exchange	Specimen mass		Water uptake		Water sample				
		Treated (mean)	Untreated	Treated (mean)	Untreated		Test water	x	y	z
	Date	g	g	g	g	no.	pH	pH	pH	pH
start										
6h						1				
24h						2				
2 d						3				
4 d						4				
8 d						5				
15 d						6				
22 d						7				
29 d						8				

Please prepare separate tables for each active ingredient

Time	Water exchange	Analytical Results													
		Untreated specimens			Treated specimens										
		Concentration a.i. in water mg/l	Quantity emitted mg/m²	Emission rate mg/m²/d	Concentration a.i. in water				Quantity emitted				Emission rate			
					x	y	z	Mean	x	y	z	Mean	x	y	z	Mean
	Date				mg/l	mg/l	mg/l	mg/l	mg/m²	mg/m²	mg/m²	mg/m²	mg/m²/d	mg/m²/d	mg/m²/d	mg/m²/d
6h																
24h																
2 d																
4 d																
8 d																
15 d																
22 d																

29 d																

Note: Since results from untreated may have to be used to correct emission rates from treated samples, the untreated results should come first and all values for treated samples would be “corrected values”. There may also be a correction for the initial water analysis.

Appendix 2

DEFINITIONS

Chemical: A substance or a mixture.

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

C.46. Bioaccumulation in Sediment-dwelling Benthic Oligochaetes

INTRODUCTION

1. This test method is equivalent to OECD test guideline (TG) 315 (2008) Sediment-ingesting endobenthic animals may be exposed to sediment bound substances (1). Among these sediment-ingesters, aquatic oligochaetes play an important role in the bottoms of the aquatic systems. They live in the sediment and often represent the most abundant species especially in habitats with environmental conditions adverse to other animals. By bioturbation of the sediment and by serving as prey these animals can have a strong influence on the bioavailability of such substances to other organisms, e.g. benthivorous fish. In contrast to epibenthic organisms, endobenthic aquatic oligochaetes burrow in the sediment, and ingest sediment particles below the sediment surface. Because of that, these organisms are exposed to substances via many uptake routes including direct contact, ingestion of contaminated sediment particles, porewater and overlying water. Some species of benthic oligochaetes that are currently used in ecotoxicological testing are described in Appendix 6.

2. The parameters which characterise the bioaccumulation of a substance include first of all the bioaccumulation factor (BAF), the sediment uptake rate constant (k_s) and the elimination rate constant (k_e). Detailed definitions of these parameters are provided in Appendix 1.

3. To assess the bioaccumulation potential of substances in general, and to investigate the bioaccumulation of substances which tend to partition into or onto the sediments, a compartment-specific test method is needed (1)(2)(3)(4).

4. This test method is designed to assess bioaccumulation of sediment-associated substances in endobenthic oligochaete worms. The test substance is spiked into the sediment. Using spiked sediment is intended to simulate a contaminated sediment.

5. This method is based on existing sediment toxicity and bioaccumulation test methods (1)(4)(5)(6)(7)(8)(9). Other useful documents are: the discussions and results of an international workshop (11), and the outcome of an international ring test (12).

6. This test applies to stable, neutral organic substances, which tend to associate with sediments. Bioaccumulation of sediment-associated, stable metallo-organic compounds can also be measured with this method (12). It is not applicable to metals and other trace elements (11) without modification of the test design with respect to substrate and water volumes, and possibly tissue sample size.

PREREQUISITE AND INFORMATION ON TEST SUBSTANCE

7. There are only a few well established Quantitative Structure-Activity Relationships (QSAR) concerning bioaccumulation processes presently available (14). The most widely used relationship is the correlation between the bioaccumulation and bioconcentration of stable organic substances and their lipophilicity (expressed as the logarithm of the octanol-water partition coefficient (log K_{ow}); see Appendix 1 for definition), respectively, which has been developed for the description of a substance partitioning between water and fish. Correlations for the sediment compartment have also been established using this relationship (15)(16)(17)(18). The log K_{ow}-log BCF correlation as a major QSAR may be helpful for a first preliminary estimation of the bioaccumulation potential of sediment-associated substances. However, the BAF may be influenced by lipid content of the test organism and the organic carbon content of the sediment. Therefore the organic carbon-water partition coefficient (K_{oc}) may also be used as a major determinant of the bioaccumulation of sediment-associated organic substances.

8. This test is applicable to:

- stable, organic substances having log K_{ow} values between 3.0 and 6.0 (5)(19) and superlipophilic substances that show a log K_{ow} of more than 6.0 (5);
- substances which belong to a class of organic substances known for their bioaccumulation potential in living organisms, e.g. surfactants or highly adsorptive substances (e.g. high K_{oc}).

9. Information on the test substance such as safety precautions, proper storage conditions and stability, and analytical methods should be obtained before beginning the study. Guidance for testing substances with physical-chemical properties that make them difficult to test is provided in (20) and (21). Before carrying out a test for bioaccumulation with aquatic oligochaetes, the following information about the test substance should be known:

- common name, chemical name (preferably IUPAC name), structural formula, CAS registry number, purity;
- solubility in water [test method A.6 (22)];
- octanol-water partition coefficient, K_{ow} [test methods A.8, A.24 (22)];
- sediment-water partition coefficient, expressed as K_d or K_{oc} [test method C.19 (22)];
- hydrolysis [test method C.7 (22)];
- phototransformation in water (23) ;
- vapour pressure [test method A.4 (22)];
- ready biodegradability [test methods C.4 and C.29 (22)];
- surface tension [test method A.5 (22)];
- critical micelles concentration (24).

In addition the following information – when available- would be relevant:

- biodegradation in the aquatic environment [test methods C.24 and C.25 (22)];
- Henry's law constant.

10. Radiolabelled test substances can facilitate the analysis of water, sediment and biological samples, and may be used to determine whether identification and quantification of degradation products should be made. The method described here was validated in an international ring test (12) for ^{14}C-labelled substances. If total

radioactive residues are measured, the bioaccumulation factor (BAF) is based on the parent substance including any retained degradation products. It is also possible to combine a metabolism study with a bioaccumulation study by analysis and quantification of the percentage of parent substance and its degradation products in samples taken at the end of the uptake phase or at the peak level of bioaccumulation. In any case, it is recommended that BAF calculation be based on the concentration of the parent substance in the organisms and not only on total radioactive residues.

11. In addition to the properties of the test substance, other information required is the toxicity to the oligochaete species to be used in the test, such as a median lethal concentration (LC_{50}) for the time necessary for the uptake phase, to ensure that selected exposure concentrations are much lower than toxic levels. If available, preference should be given to toxicity values derived from long-term studies on sublethal endpoints (EC_{50}). If such data are not available, an acute toxicity test under conditions identical with the bioaccumulation test conditions, or toxicity data on other surrogate species data may provide useful information.

12. An appropriate analytical method of known accuracy, precision, and sensitivity for the quantification of the substance in the test solutions, in the sediment, and in the biological material must be available, together with details of sample preparation and storage as well as material safety data sheets. Analytical detection limits of the test substance in water, sediment, and worm tissue should also be known. If a radiolabelled test substance is used, the specific radioactivity (i.e. Bq mol^{-1}), the position of the radiolabelled atom, and the percentage of radioactivity associated with impurities must also be known. The specific radioactivity of the test substance should be as high as possible in order to detect test concentrations as low as possible (11).

13. Information on characteristics of the sediment to be used (e.g. origin of sediment or its constituents, pH and ammonia concentration of the pore water (field sediments), organic carbon content (TOC), particle size distribution (per cent sand, silt, and clay), and per cent dry weight) should be available (6).

PRINCIPLE OF THE TEST

14. The test consists of two phases; the uptake (exposure) phase and the elimination (post-exposure) phase. During the uptake phase, worms are exposed to sediment spiked with the test substance, topped with reconstituted water and equilibrated as appropriate (11). Groups of control worms are held under identical conditions without the test substance.

15. For the elimination phase the worms are transferred to a sediment-water-system free of test substance. An elimination phase is necessary to gain information on the rate at which the test substance is excreted by the test organisms (19)(25). An elimination phase is always required unless uptake of the test substance during the exposure phase has been insignificant (e.g. there is no statistical difference between the concentration of the test substance in test and control worms). If a steady state has not been reached during the uptake phase, determination of the kinetics – BAF_k, uptake and elimination rate constant(s) – may be done using the results of the elimination phase. Change of

the concentration of the test substance in/on the worms is monitored throughout both phases of the test.

16. During the uptake phase, measurements are made until BAF has reached a plateau or steady state. By default, the duration of the uptake phase should be 28 days. Practical experience has shown that a 12 to 14-day uptake phase is sufficient for several stable, neutral organic substances to reach steady-state (6)(8)(9).

17. However, if the steady state is not reached within 28 d, the elimination phase is started by transferring exposed oligochaetes to vessels containing the same medium without the test substance. The elimination phase is terminated when either the 10% level of the concentration measured in the worms on day 28 of the uptake phase is reached, or after a maximum duration of 10 d. The residue level in the worms at the end of the elimination phase is reported as an additional endpoint, e.g. as Non-eliminated residues (NER). The bioaccumulation factor (BAF_{ss}) is calculated preferably both as the ratio of concentration in worms (C_a) and in the sediment (C_s) at apparent steady state, and as a kinetic bioaccumulation factor, BAF_K as the ratio of the rate constant of uptake from sediment (k_s) and the elimination rate constant (k_e) assuming first-order kinetics. If a steady state is not reached within 28 days, calculate BAF_K from the uptake rate and elimination rate constant(s). For calculation see Appendix 2. If first-order kinetics are not applicable, more complex models should be employed (Appendix 2 and reference (25).

18. If a steady state is not achieved within 28 days, the uptake phase may optionally be extended subjecting groups of exposed worms – if available – to further measurements until steady state is reached; in parallel, the elimination phase should nevertheless be started on day 28 of the uptake phase.

19. The uptake rate constant, the elimination rate constant (or constants, where more complex models are involved), the kinetic bioaccumulation factor (BAF_K), and where possible, the confidence limits of each of these parameters are calculated from computerised model equations (see Appendix 2 for models). The goodness of fit of any model can be determined from the correlation coefficient or the coefficient of determination (coefficients close to 1 indicate a good fit).

20. To reduce variability in test results for organic substances with high lipophilicity, bioaccumulation factors should be expressed additionally in relation to the lipid content of the test organisms and to the organic carbon content (TOC) in the sediment (biota-sediment accumulation factor or BSAF in kg sediment TOC kg^{-1} worm lipid content). This approach is based on experiences and theoretical correlations for the aquatic compartment, where – for some chemical classes – there is a clear relationship between the potential of a substance to bioaccumulate and its lipophilicity, which has been well established for fish as model organisms (14)(25)(27). There is also a relationship between the lipid content of the test fish and the observed bioaccumulation of such substances. For benthic organisms, similar correlations have been found (15)(16)(17)(18). If sufficient worm tissue is available, the lipid content of the test animals may be determined on the same biological material as the one used to determine the concentration of the test substance. However, it is practical to use acclimatised control animals at least at start or – preferably – at the end of the uptake

phase to measure the lipid content, which can then be used to normalise the BAF values.

VALIDITY OF THE TEST

21. For a test to be valid the following conditions apply:

- The cumulative mortality of the worms (controls and treatments) until the end of the test should not exceed 20% of the initial number.
- In addition, it should be demonstrated that the worms burrow in the sediment to allow for maximum exposure. For details see paragraph 28.

DESCRIPTION OF THE METHOD

Test species

22. Several species of aquatic oligochaetes can be used for the test. The most commonly used species are listed in Appendix 6.

23. Toxicity tests (96 h, in water only) should be conducted at regular intervals (e.g. every month) with a reference toxicant such as potassium chloride (KCl) or copper sulfate ($CuSO_4$) (1) to demonstrate the health conditions of the test animals (1)(6). If reference toxicity tests are not conducted at regular intervals, the batch of organisms to be used in a sediment bioaccumulation test should be checked using a reference toxicant. Measurement of the lipid content might also provide useful information on the condition of the animals.

Culture of the test organisms

24. In order to have a sufficient number of worms for conducting bioaccumulation tests the worms may have to be kept in permanent single-species laboratory culture. Laboratory culture methods for the selected test species are summarised in Appendix 6. For details see references (8)(9)(10)(18)(28)(29)(30)(31)(32).

Apparatus

25. Care should be taken to avoid the use of materials for all parts of the equipment that can dissolve, absorb test substances or leach other substances and have an adverse effect on the test animals. Standard rectangular or cylindrical chambers, made of chemically inert material and of suitable capacity in compliance with the loading rate, i.e. the number of test worms can be used. The use of soft plastic tubing for administering water or air should be avoided. Polytetrafluoroethylene, stainless steel and/or glass should be used for any equipment having contact with the test media. For substances with high adsorption coefficients, such as synthetic pyrethroids, silanised glass may be required. In these situations the equipment will have to be discarded after use (5). For radiolabelled test substances, and for volatile substances, care should be taken to avoid stripping and the escape of stripped test substance. Traps (e.g. glass gas washing bottles) containing suitable absorbents to retain any residues evaporating from

the test chambers should be employed (11).

Water

26. The overlying water must be of a quality that will allow the survival of the test species for the duration of the acclimation and test periods without them showing any abnormal appearance or behaviour. Reconstituted water according to test method C.1 (25) is recommended for use as overlying water in the tests as well as in the laboratory cultures of the worms. It has been demonstrated that several test species can survive, grow, and reproduce in this water (8), and maximum standardisation of test and culture conditions is provided. The water should be characterised at least by pH, conductivity and hardness. Analysis of the water for micro-pollutants prior to use might provide useful information (Appendix 4).

27. The water should be of constant quality during the period of a test. The pH of the overlying water should be between 6 and 9. The total hardness should be between 90 and 400 mg $CaCO_3$ per litre at the start of the test (7). Ranges for pH and hardness in the mentioned reconstituted water are given in test method C.1 (25). If there is an interaction suspected between hardness ions and the test substance, lower hardness water should be used. Appendix 4 summarises additional criteria of an acceptable dilution water according to OECD TG 210 (34).

Sediment

28. The sediment must be of a quality that will allow the survival and preferably the reproduction of the test organisms for the duration of the acclimation and test periods without them showing any abnormal appearance or behaviour. The worms should burrow into the sediment. Burrowing behaviour can have an influence on the exposure, and consequently on the BAF. Therefore, sediment avoidance or burrowing behaviour of the test organisms should be recorded, where turbidity of the overlying water allows such observations. The worms (control and treatments) should burrow in the sediment within a period of 24 h after addition to the test vessels. If permanent burrowing failure or sediment avoidance are observed (e.g. more than 20% over more than half of the uptake phase), this indicates that either the test conditions are not appropriate, or the test organisms are not healthy, or that the concentration of the test substance elicits this behaviour. In such a case the test should be stopped and repeated at improved conditions. Additional information on sediment ingestion can be obtained by using methods described in (35)(36), which specify sediment ingestion or particle selection in the test organisms. If observable, at least the presence or absence of fecal pellets on the sediment surface, which indicate sediment ingestion by the worms, should be recorded and considered for the interpretation of the test results with respect to exposure pathways.

29. An artificial sediment based on the artificial soil described in test method C.8 (40) is recommended for use in both the tests and the laboratory cultures of the worms (Appendix 5), since natural sediments of appropriate quality may not be available throughout the year. In addition, indigenous organisms as well as the possible presence of micropollutants in natural sediments might influence the test. Several test species can survive, grow, and reproduce in the artificial sediment (8).

30. The artificial sediment should be characterised at least by origin of the constituents, grain size distribution (percent sand, silt, and clay), organic carbon content (TOC), water content, and pH. Measurement of redox potential is optional. However, natural sediments from unpolluted sites may serve as test and/or culture sediment (1). Natural sediments should be characterised at least by origin (collection site), pH and ammonia of the pore water, organic carbon content (TOC), particle size distribution (percent sand, silt, and clay), and percent water content (6). It is recommended that, before it is spiked with the test substance, the natural sediment be conditioned for seven days under the same conditions which prevail in the subsequent test, if ammonia development is expected. At the end of this conditioning period, the overlying water should be removed and discarded. Analysis of the sediment or its constituents for micro-pollutants prior to use might provide useful information.

Preparation

31. Handling of natural sediments prior to their use in the laboratory is described in (1)(6)(44). The preparation of the artificial sediment is described in Appendix 5.

Storage

32. The storage of natural sediments in the laboratory should be as short as possible. U.S. EPA (6) recommends a maximum storage period of 8 weeks at $4 \pm 2°C$ in the dark. There should be no headspace above the sediment in the storage containers. Recommendations for the storage of artificial sediment are given in Appendix 5.

Application of the test substance

33. The sediment is spiked with the test substance. The spiking procedure involves coating of one or more of the sediment constituents with the test substance. For example, the quartz sand, or a portion thereof (e.g. 10 g of quartz sand per test vessel), can be soaked with a solution of the test substance in a suitable solvent, which is then slowly evaporated to dryness. The coated fraction can then be mixed into the wet sediment. The amount of sand provided by the test-substance-and-sand mixture has to be taken into account when preparing the sediment, i.e. the sediment should thus be prepared with less sand (6).

34. With a natural sediment, the test substance may be added by spiking a dried portion of the sediment as described above for the artificial sediment, or by stirring the test substance into the wet sediment, with subsequent evaporating of any solubilising agent used. Suitable solvents for spiking wet sediment are ethanol, methanol, ethylene glycol monomethyl ether, ethylene glycol dimethyl ether, dimethylformamide and triethylene glycol (5)(34). Toxicity and volatility of the solvent and the solubility of the test substance in the chosen solvent should be the main criteria for the selection of a suitable solubilising agent. Additional guidance on spiking procedures is given in Environment Canada (1995)(41). Care should be taken to ensure that the test substance added to sediment is thoroughly and evenly distributed within the sediment. Replicated sub-samples of the spiked sediment should be analysed to check the concentrations of the test substance in the sediment, and to determine the degree of homogeneity of test substance distribution.

35. Once the spiked sediment with overlying water has been prepared, it is desirable to allow partitioning of the test substance between the sediment and the aqueous phase. This should preferably be done under the conditions of temperature and aeration used in the test. Appropriate equilibration time is sediment and substance specific, and can be in the order of hours to days and in rare cases up to several weeks (4-5 weeks) (28)(42). In this test, equilibrium is not awaited but an equilibration period of 48 hours to 7 days is recommended. Depending on the purpose of the study, e.g., when environmental conditions are to be mimicked, the spiked sediment may be equilibrated or aged for a longer period (11).

PERFORMANCE OF THE TEST

Preliminary test

36. It may be useful to conduct a preliminary experiment in order to optimise the test conditions of the definitive test, e.g. selection of test substance concentration(s) and duration of the uptake and elimination phases. The behaviour of worms, for example sediment avoidance, i.e. the worms escape from the sediment which may be caused by the test substance and/or by the sediment itself, should be observed and recorded during a preliminary test. Sediment avoidance may also be used as a sub-lethal parameter in a preliminary test for estimating the test substance concentration(s) to be used in a bioaccumulation test.

Exposure conditions

Duration of the uptake phase

37. The test organisms are exposed to the test substance during the uptake phase. The first sample should be taken between 4 and 24 h after start of uptake phase. The uptake phase should be run for up to 28 days (1)(6)(11) unless it can be demonstrated that equilibrium has been reached earlier. The steady state occurs when: (i) a plot of the bioaccumulation factors at each sampling period against time is parallel to the time axis; (ii) three successive analyses of BAF made on samples taken at intervals of at least two days vary no more than ± 20% of each other; and (iii) there are no significant differences between the three sampling periods (based on statistical comparisons e.g. analysis of variance and regression analysis). If the steady state has not been reached by 28 days, the uptake phase may be ended by starting the elimination phase, and the BAF_K can be calculated from the uptake and elimination rate constants (see also paragraphs 16 to 18).

Duration of the elimination phase

38. The first sample should be taken between 4 and 24 h after start of elimination phase, since during the initial period, rapid changes in tissue residue may occur. It is recommended to terminate the elimination phase either when the concentration of test substance is less than 10% of steady-state concentration, or after a maximum duration of 10 days. The residue level in the worms at the end of the elimination phase is reported as a secondary endpoint. The period may, however, be governed by the period over which the concentration of the test substance in the worms remains above the

analytical detection limit.

Test organisms

Numbers of test worms

39. The number of worms per sample must provide a mass of worm tissue such that the mass of test substance per sample at the beginning of the uptake phase and at the end of the elimination phase, respectively, is significantly higher than the detection limit for the test substance in biological material. In the mentioned stages of uptake and elimination phases the concentration in the test animals is usually relatively low (6)(8)(18). Since the individual weight in many species of aquatic oligochaetes is very low (5-10 mg wet weight per individual for *Lumbriculus variegatus* and *Tubifex tubifex*), the worms of a given replicate test chamber may be pooled for weighing and test chemical analysis. For test species with higher individual weight (e.g. *Branchiura sowerbyi*) replicates containing one individual may be used, but in such cases the number of replicates should be increased to five per sampling point (11). It should however be noted that *B. sowerbyi* was not included in the ring test (12), and is therefore not recommended as a preferable species in the method.

40. Worms of similar size should be used (for *L. variegatus* see Appendix 6). They should come from the same source, and should be adult or large animals of the same age class (see Appendix 6). The weight and age of an animal may have a significant effect on the BAF-values (e.g. due to different lipid content and/or presence of eggs); these parameters should be recorded accurately. To measure the mean wet and dry weight a sub-sample of worms should be weighed before starting the test.

41. With *Tubifex tubifex* and *Lumbriculus variegatus*, reproduction is expected during the test period. A lack of reproduction in a bioaccumulation test should be recorded, and considered when interpreting the test results.

Loading

42. High sediment-to-worm and water-to-worm ratios should be used in order to minimise the reduction of test substance concentration in the sediment during the uptake phase, and to avoid decreases in dissolved oxygen concentration. The chosen loading rate should also correspond to naturally occurring population densities of the chosen species (43). For example, for *Tubifex tubifex*, a loading rate of 1-4 mg of worm tissue (wet weight) per gram of wet sediment is recommended (8)(11). References (1) and (6) recommend a loading rate of ≤1 g dry weight of worm tissue per 50 g sediment organic carbon for *L. variegatus*.

43. The worms to be used in a test are removed from the culture by sieving the culture sediment. The animals (adult or large worms without signs of recent fragmentation) are transferred to glass dishes (e.g. petri dishes) containing clean water. If the test conditions differ from the culture conditions, an acclimation phase of 24 h should be sufficient. Prior to weighing, excess water should be removed from the worms. This can be done by gently placing the worms on a pre-moistened paper tissue. It is not recommended to use absorbing paper to dry the worms as this may cause stress or

damage to the worms. Brunson et al. (1998) recommend using non-blotted worms of approximately 1.33 times the target biomass. These additional 33% correspond to the difference between blotted and non-blotted worms (28).

44. At the start of the uptake phase (day 0 of the test), the test organisms are removed from the acclimatisation chamber and distributed randomly to vessels (e.g. petri dishes) containing reconstituted water by adding groups of two worms to each vessel, until each vessel contains ten worms. Each of these groups of worms are then randomly transferred to separate test vessels, e.g. using soft steel forceps. The test vessels are subsequently incubated under test conditions.

Feeding

45. In view of the low nutrient content of the artificial sediment, the sediment should be amended with a food source. In order not to underestimate the exposure of the test organisms, e.g. by selectively feeding uncontaminated food, the food necessary for reproduction and growth of the test organisms should be added to the sediment once before or during application of the test substance (see Appendix 5).

Sediment-water ratio

46. The recommended sediment-water ratio is 1:4 (45). This ratio is considered suitable to maintain oxygen concentrations at appropriate levels, and to avoid the build-up of ammonia in the overlying water. The oxygen content in the overlying water should be maintained at $\geq$ 40% saturation. The overlying water of the test vessels should be gently aerated (e.g. 2 – 4 bubbles per second) via a pasteur pipette positioned approximately 2 cm above the sediment surface so as to minimise perturbation of the sediment.

Light and temperature

47. The photoperiod in the culture and the test is 16 hours (1)(6). Light intensity in the test area should be kept at about 500-1000 lx. The temperature should be 20 ± 2°C throughout the test.

Test concentrations

48. One test concentration (as low as possible) is used for determination of the uptake kinetics, but a second (higher) concentration may be used (e.g. (46)). In that case, samples are taken and analysed at steady state or after 28 d to confirm the BAF measured at the lower concentration (11). The higher concentration should be selected so that adverse effects can be excluded (e.g. by choosing approximately 1% of the lowest known chronic effect concentration EC_x as derived from relevant chronic toxicity studies). The lower test concentration should be significantly higher than the detection limit in sediment and biological samples by the analytical method used. If the effect concentration of the test substance is close to the analytical detection limit, the use of radiolabelled test substance with high specific radioactivity is recommended.

Treated and Control Replicates

49. The minimum number of treated replicates for kinetic measurements should be three per sampling point (11) throughout uptake and elimination phase. Additional replicates should be employed e.g. for optional additional sampling dates. For the elimination phase, a matching number of replicates is prepared with non-spiked sediment and overlying water, so that the treated worms can be transferred from designated treated vessels to non-treated vessels at the end of the uptake phase. The total number of treated replicates should be sufficient for both uptake and elimination phase.

50. Alternatively, the worms designated for sampling during the elimination phase may be exposed in one large container containing spiked sediment of the same batch as used for uptake kinetics. It should be demonstrated that the test conditions (e.g. sediment depth, sediment water ratio, loading, temperature, water quality) are comparable to the replicates designated for the uptake phase. At the end of the uptake phase, water, sediment and worm samples should be taken from this container for analysis, and a sufficient number of large worms that show no sign of recent fragmentation, should be removed carefully and transferred to the replicates prepared for the elimination phase (e.g. ten organisms per replicate vessel).

51. If no solvent other than water is used, at least 9 replicates of a negative control (at least 3 sampled at start, 3 at end of uptake and 3 at end of elimination) should be provided for biological and background analysis. If any solubilising agent is used for application of the test substance, a solvent control should be run (at least 3 replicates should be sampled at start, 3 at the end of the uptake phase, and 3 at the end of the elimination phase). In this case, at least 4 replicates of a negative control (no solvent) should be provided for sampling at the end of the uptake phase. These replicates can be compared biologically with the solvent control in order to gain information on possible influence of the solvent on the test organisms. Details are given in Appendix 3.

Frequency of water quality measurements

52. As a minimum, the following water quality parameters should be measured in the overlying water during uptake and elimination phase:

• Temperature	in one vessel of each treatment level per sampling date, and in one control vessel once per week and at the start and the end of the uptake and elimination period; temperature in the surrounding medium (ambient air or water bath) or in one representative test vessel may also be recorded e.g. in continuous or hourly intervals;
• Dissolved oxygen content	in one vessel of each treatment level, and in one control vessel per sampling date; expressed as mg/L and % ASV (air saturation value);
• Air supply	controlled at least once per day (workdays) and adjusted if needed;

• pH	in one treated vessel of each treatment level per sampling date, and in one control vessel once per week and at the start and the end of the uptake and elimination period;
• Total water hardness	at least in one treated vessel and one control test vessel at the start and the end of the uptake and elimination period, expressed as mg/l $CaCO_3$;
• Total ammonia content	at least in one treated vessel and one control test vessel at the start and the end of the uptake and elimination period; expressed as mg/l NH_4^+ or NH_3 or total ammonia-N.

Sampling and analysis of worms, sediment, and water

Sampling Schedule

53. Examples of sampling schedules for a 28-day uptake phase and a 10-day elimination phase are given in Appendix 3.

54. Sample the water and sediment from the test chambers for determination of test substance concentration before adding the worms, and during both uptake and elimination phases. During the test the concentrations of test substance are determined in the worms, sediment, and water in order to monitor the distribution of the test substance in the compartments of the test system.

55. Sample the worms, sediment, and water on at least six occasions during the uptake as well as the elimination phase.

56. Continue sampling until a plateau (steady state) has been established (see Appendix 1) or for 28 days. If the plateau has not been reached within 28 days, begin the elimination phase. When beginning the elimination phase, transfer the designated worms to replicate chambers containing untreated sediment and water (see also paragraphs 17 and 18).

Sampling and sample preparation

57. Obtain water samples by decanting, siphoning or pipetting a volume sufficient for measuring the quantity of the test substance in the sample.

58. The remaining overlying water is carefully decanted or siphoned from the test chamber(s). Sediment samples should be taken carefully, causing minimal disturbance of the worms.

59. Remove all worms from the test replicate at the sampling time, e.g. by suspending the

sediment with overlying water and spreading the contents of each replicate on a shallow tray and picking the worms using soft steel forceps. Rinse them quickly with water in a shallow glass or steel tray. Remove the excess water. Transfer the worms carefully to a pre-weighed vessel and weigh them. Sacrifice the worms by freezing (e.g. ≤ - 18 °C). The presence and number of cocoons and/or juveniles should be recorded.

60. In general, the worms should be weighed and sacrificed immediately after sampling without a gut purging phase to obtain a conservative BAF which includes contaminated gut content, and to avoid losses of body residues during any gut-purging period in water only (8). Substances with log K_{ow} above 5 are not expected to be eliminated significantly during any gut-purging period in water only, while substances with log Kow lower than 4 may be lost in notable amounts (47).

61. During the elimination phase, the worms purge their gut in clean sediment. This means, measurements immediately before the elimination phase include contaminated gut sediment, while after the initial 4-24 h of the elimination phase, most of the contaminated gut content is assumed to be replaced by clean sediment (11)(47). The concentration in the worms of this sample may then be considered as the tissue concentration after gut purge. To account for dilution of the test substance concentration by uncontaminated sediment during the elimination phase, the weight of the gut content may be estimated from worm wet weight/worm ash weight or worm dry weight/worm ash weight ratios.

62. If the purpose of a specific study is to measure the bioavailability and true tissue residues in the test organisms, then at least a sub-sample of treated animals (e.g. from three additional replicate vessels), preferably sampled during steady state, should be weighed, purged in clean water for a period of 6 hours (47), and weighed again before analysis. Data on worm weight and body concentration of this sub-sample can then be compared to values obtained from un-purged worms. The worms designated for measurement of elimination should not be purged before the transfer to clean sediment to minimise additional stress for the animals.

63. Preferably analyse the water, sediment, and worm samples immediately (i.e. within 1-2 d) after removal in order to prevent degradation or other losses and to calculate the approximate uptake and elimination rates as the test proceeds. Immediate analysis also avoids delay in determining when a plateau has been reached.

64. Failing immediate analysis, the samples should be stored under appropriate conditions. Obtain information on the stability and proper storage conditions for the particular test substance before beginning the study, (e.g. duration and temperature of storage, extraction procedures, etc.). If such information is not available and it is judged to be necessary, spiked control tissues can be run concurrently to determine storage stability.

Quality of analytical method

65. Since the whole procedure is governed essentially by the accuracy, precision and sensitivity of the analytical method used for the test substance, check experimentally

that the precision and reproducibility of the chemical analysis, as well as the recovery of the test substance from water, sediment and worm samples are satisfactory for the particular method. Also, check that the test substance is not detectable in the control chambers in concentrations higher than background. If necessary, correct the values of C_w, C_s and C_a for the recoveries and background values of controls. Handle all samples throughout the test in such a manner so that contamination and loss are minimised (e.g. resulting from adsorption of the test substance on the sampling device).

66. The overall recovery and the recovery of test substance in worms, sediment, water, and, if employed, in traps containing absorbents to retain evaporated test substance, should be recorded and reported.

67. Since the use of radiolabelled substances is recommended, it is possible to analyse for total radioactivity (i.e. parent and degradation products). However, if analytically feasible, quantification of parent substance and degradation products at steady state or at the end of the uptake phase can provide important information. If it is intended to perform such measurements, the samples should then be subjected to appropriate extraction procedures so that the parent substance can be quantified separately. Where a detected degradation product represents a significant percentage (e.g. > 10%) of the radioactivity measured in the test organisms at steady state or at the end of the uptake phase, it is recommended to identify such degradation products (5).

68. Due to low individual biomass, it is often not possible to determine the concentration of test substance in each individual worm, unless Branchiura sowerbyi (40-50 mg wet weight per worm) is used as test species (11). Therefore, pooling of the individuals sampled from a given test vessel is acceptable, but it does restrict the statistical procedures which can be applied to the data. If a specific statistical procedure and power are important considerations, then an adequate number of test animals and/or replicate test chambers to accommodate the desired pooling, procedure and power, should be included in the test.

69. It is recommended that the BAF is expressed both as a function of total wet weight, total dry weight, and, when required (e.g. for highly lipophilic substances) as a function of the lipid content and the TOC of the sediment. Suitable methods should be used for determination of lipid content (48)(49). The chloroform/methanol extraction technique (50) may be recommended as standard method (48). However, to avoid the use of chlorinated solvents, a ring-tested modification of the Bligh & Dyer method (50) as described in (51) might be used. Since the various methods do not give identical values (48), it is important to detail the method used. When possible, i.e. if sufficient worm tissue is available, the lipid content is measured in the same sample or extract as that produced for analysis for the test substance, since the lipids often have to be removed from the extract before it is analysed by chromatography (5). However, it is practical to use acclimatised control animals at least at start or - preferably - at the end of the uptake phase to measure the lipid content, e.g. in three samples.

DATA AND REPORTING

Treatment of results

70. The uptake curve of the test substance is obtained by plotting in arithmetic scale the concentration of test substance in/on the worms during the uptake phase against time. If the curve has reached a plateau, calculate the steady state BAF_{ss}:

$$\frac{C_a \text{ at steady state or at day 28 (mean)}}{C_s \text{ at steady state or at day 28 (mean)}}$$

71. Determine the kinetic bioaccumulation factor (BAFK) as the ratio ks/ke. The elimination constant (ke) is usually determined from the elimination curve (i.e. a plot of the concentration of the test substance in the worms during the elimination phase). The uptake rate constant ks is then calculated from the uptake curve kinetics. The preferred method for obtaining BAFK and the rate constants, ks, and ke, is to use non-linear parameter estimation methods on a computer (see Appendix 2). If the elimination is obviously not first-order, then more complex models should be employed (25)(27)(52).

72. The biota-sediment accumulation factor (BSAF) is determined by normalising the BAFK for the worm lipid content and the sediment total organic carbon content.

Interpretation of results

73. The results should be interpreted with caution where measured concentrations of test concentrations occur at levels close to the detection limit of the analytical method used.

74. Clearly defined uptake and elimination curves are an indication of good quality bioaccumulation data. Generally the confidence limits for the BAF values from well-designed studies should not exceed 25% (5).

Test report

75. The test report must include the following information.

Test substance
- physical nature and, physicochemical properties e.g. log K_{ow}, water solubility;
- chemical identification data; source of the test substance, identity and concentration of any solvent used;
- if radiolabelled, the precise position of the labelled atoms, the specific radioactivity, and the percentage of radioactivity associated with impurities.

Test species
- scientific name, strain, source, any pre-treatment, acclimation, age, size-range, etc..

Test conditions
- test procedure used (e.g. static, semi-static or flow-through);
- type and characteristics of illumination used and photoperiod(s);
- test design (e.g. number, material and size of test chambers, water volume, sediment mass and volume, water volume replacement rate (for flow-through or semi-static

procedures), any aeration used before and during the test, number of replicates, number of worms per replicate, number of test concentrations, length of uptake and elimination phases, sampling frequency);
- method of test substance preparation and application as well as reasons for choosing a specific method;
- the nominal test concentrations;
- source of the constituents of the artificial water and sediment or - if natural media are used - origin of the water and the sediment, description of any pre-treatment, results of any demonstration of the ability of the test animals to live and/or reproduce in the media used, sediment characteristics (pH and ammonia of the pore water (natural sediments), organic carbon content (TOC), particle size distribution (percent sand, silt, and clay), percent water content, and any other measurements made) and water characteristics (pH, hardness, conductivity, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), and any other measurements made);
- the nominal and measured dry weight in % of wet weight (or dry weight-to-wet weight ratio) of the artificial sediment; the measured dry weight in % of wet weight (or dry weight-to-wet weight ratio) for field sediments;
- water quality within the test chambers as characterised by temperature, pH, ammonium, total hardness, and dissolved oxygen concentration;
- detailed information on the treatment of water, sediment, and worm samples, including details of preparation, storage, spiking procedures, extraction, and analytical procedures (and precision) for the test substance and lipid content, and recoveries of the test substance.

Results
- mortality of the control worms and the worms in each test chamber and any observed sublethal effects including abnormal behaviour (e.g., sediment avoidance, presence or absence of fecal pellets, lack of reproduction);
- the measured dry weight in % of wet weight (or dry weight-to-wet weight ratio) of the sediment and the test organisms (useful for normalisation);
- the lipid content of the worms;
- curves showing the uptake and elimination kinetics of the test substance in the worms, and the time to steady state;
- C_a, C_s and C_w (with standard deviation and range, if appropriate) for all sampling times (C_a expressed in g kg^{-1} wet and dry weight of whole body, C_s expressed in g kg^{-1} wet and dry weight of sediment, and C_w in mg l^{-1}). If a biota-sediment accumulation factor (BSAF; see Appendix 1 for definition) is required (e.g. for comparison of results from two or more tests performed with animals of differing lipid content), C_a should additionally be expressed as g kg^{-1} lipid content of the organism, and C_s should be expressed as g kg^{-1} organic carbon (OC) of the sediment;
- BAF (expressed in kg wet sediment kg^{-1} wet worm), sediment uptake rate constant k_s (expressed in g wet sediment kg^{-1} of wet worm d^{-1}), and elimination rate constant k_e (expressed in d^{-1}); BSAF (expressed in kg sediment OC kg^{-1} worm lipid content) may be reported additionally;
- Non-eliminated residues (NER) at end of elimination phase;
- if measured: percentages of parent substance, degradation products, and bound residues (i.e. the percentage of test substance that cannot be extracted with common extraction methods) detected in the test animals;
- methods used for statistical analyses of the data.

Evaluation of results

- compliance of the results with the validity criteria as listed in paragraph 21;
- unexpected or unusual results, e.g. incomplete elimination of the test substance from the test animals; in such cases results from any preliminary study may provide useful information.

APPENDIX 1

DEFINITIONS AND UNITS

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

Bioaccumulation is the increase in concentration of the test substance in or on an organism relative to the concentration of the test substance in the surrounding medium. Bioaccumulation results from both bioconcentration and biomagnification processes (see below).

The **bioaccumulation factor** (BAF) at any time during the uptake phase of this bioaccumulation test is the concentration of test substance in/on the test organism (C_a in g kg^{-1} wet or dry weight) divided by the concentration of the substance in the surrounding medium (C_s as g kg^{-1} of wet or dry weight of sediment). In order to refer to the units of C_a and C_s, the BAF has the units of kg sediment kg^{-1} worm (15).

Bioaccumulation factors calculated directly from the ratio of the sediment uptake rate constant divided by the elimination rate constants (k_s and k_e, respectively - see below) are termed kinetic bioaccumulation factor (BAF_K).

Bioconcentration is the increase in concentration of the test substance in or on an organism, resulting exclusively from uptake via the body surface, relative to the concentration of the test substance in the surrounding medium.

Biomagnification is the increase in concentration of the test substance in or on an organism, resulting mainly from uptake from contaminated food or prey, relative to the concentration of the test substance in the food or prey. Biomagnification can lead to a transfer or accumulation of the test substance within food webs.

The **biota-sediment accumulation factor** (BSAF) is the lipid-normalised steady state concentration of test substance in/on the test organism divided by the organic carbon-normalised concentration of the substance in the sediment at steady state. C_a is then expressed as g kg^{-1} lipid content of the organism, and C_s as g kg^{-1} organic content of the sediment.

The **conditioning period** is used to stabilise the microbial component of the sediment and to remove e.g. ammonia originating from sediment components; it takes place prior to spiking of the sediment with the test substance. Usually, the overlying water is discarded after conditioning.

The **elimination** of a test substance is the loss of this substance from the test organism tissue by active or passive processes that occurs independently of presence or absence of the test substance in the surrounding medium.

The **elimination phase** is the time, following the transfer of the test organisms from a contaminated medium to a medium free of the test substance, during which the elimination (or the net loss) of the substance from the test organisms is studied.

The **elimination rate constant** (k_e) is the numerical value defining the rate of reduction in the concentration of the test substance in/on the test organism, following the transfer of the test organisms from a medium containing the test substance to a chemical-free medium; k_e is expressed in d^{-1}.

The **equilibration period** is used to allow for distribution of the test substance between the solid phase, the pore water and the overlying water; it takes place after spiking of the sediment with the test substance and prior to addition of the test organisms.

The **octanol-water partitioning coefficient** (K_{ow}) is the ratio of substance´s solubility in n-octanol and in water at equilibrium, also sometimes expressed as P_{ow}. The logarithm of K_{ow} (log K_{ow}) is used as an indication of a substance´s potential for bioaccumulation by aquatic organisms.

The **organic carbon-water partitioning coefficient** (K_{oc}) is the ratio of a substance´s concentration in/on the organic carbon fraction of a sediment and the substance´s concentration in water at equilibrium.

Overlying water is the water lying on top of the sediment in the test vessel.

A **plateau** or **steady state** is defined as the equilibrium between the uptake and elimination processes that occur simultaneously during the exposure phase. The steady state is reached in the plot of the BAF at each sampling period against time when the curve becomes parallel to the time axis and three successive analyses of BAF made on samples taken at intervals of at least two days are within 20% of each other, and there are no statistically significant differences among the three sampling periods. For test substances which are taken up slowly, more appropriate intervals would be seven days (5).

Pore water or interstitial water is the water occupying space between sediment or soil particles.

The **sediment uptake rate constant** (k_s) is the numerical value defining the rate of increase in the concentration of the test substance in/on the test organism resulting from uptake from the sediment phase. k_s is expressed in g sediment kg^{-1} of worm d^{-1}.

Spiked sediment is sediment to which test substance has been added.

The **steady state bioaccumulation factor** (BAF_{ss}) is the BAF at steady state and does not change significantly over a prolonged period of time, the concentration of the test substance in the surrounding medium (C_s as g kg^{-1} of wet or dry weight of sediment) being constant during this period of time.

The **uptake or exposure phase** is the time during which the test organisms are exposed to the test substance.

APPENDIX 2

CALCULATION OF UPTAKE AND ELIMINATION PARAMETERS

The main endpoint of a bioaccumulation test is the bioaccumulation factor, BAF. The measured BAF can be calculated by dividing the concentration of the test substance in the test organism, C_a, by the concentration of the test substance in the sediment, C_s, at steady state. If the steady state is not reached during the uptake phase, the BAF is calculated in the same manner for day 28. However, it should be noted whether the BAF is based on steady state concentrations or not.

The preferred means for obtaining the kinetic bioaccumulation factor (BAF_K), the sediment uptake rate constant (k_s) and the elimination rate constant (k_e) is to use non-linear parameter estimation methods on a computer. Given the time series of average accumulation factors (C_a, mean values of each sampling date/C_s, mean values of each sampling date = AF) of the uptake phase based on worm and sediment wet weight, and the model equation

$$AF(t) = BAF \times (1\text{-}e^{ke \times t}) \qquad \text{[equation 1]}$$

where AF(t) is the ratio of concentration of the test substance in worms and its concentration in the sediment at any given time point (t) of the uptake phase, these computer programs calculate values for BAF_K, k_s and k_e.

When steady state is reached during the uptake phase (i.e. $t = \infty$), equation 1 may be reduced to:

$$BAF_K = \frac{k_s}{k_e} \qquad \text{[equation 2]}$$

where k_s = uptake rate constant in tissue [g sediment kg^{-1} of worm d^{-1}]
k_e = elimination rate constant [d^{-1}]

Then $k_s/k_e \times C_s$ is an approach to the concentration of the test substance in the worm tissue at steady state ($C_{a,ss}$).

The Biota-Sediment Accumulation Factor (BSAF) should be calculated as follows:

$$BSAF = BAF_K \times \frac{f_{oc}}{f_{lip}}$$

where foc is the fraction of sediment organic carbon, and flip is the fraction of worm lipid, both based either on dry weight, or on wet weight.

Given a time series of concentration values, the elimination kinetics can be modelled using the following model equations and a computer calculation based non-linear parameter estimation method.

The mean measured body residue at the end of the uptake phase is recommended as the default starting point. The value modeled/estimated from the uptake phase should only be used, e.g. if the measured value deviates significantly from the modelled body residue. See also paragraph 50 for alternative pre-exposure of worms designated for elimination; with this approach, samples of these pre-exposed worms on day 0 of the elimination phase are thought to provide a realistic body residue to start the elimination kinetics with.

If the data points plotted against time indicate a constant exponential decline of the test substance concentration in the animals, a one-compartment model (equation 4) can be used to describe the time course of elimination.

$$C_a(t) = C_{a,ss} \times e^{-k}e^{t} \qquad \text{[equation 3]}$$

Elimination processes sometimes appear to be biphasic, showing a rapid decline of C_a during the early phases, that changes to a slower loss of test substances in the later phases of the elimination (8)(19)(25)). The two phases can be interpreted by the assumption, that there are two different compartments in the organism, from which the test substance is lost with different velocity. In these cases specific literature should be studied (15)(16)(17)(25).

A two-compartment elimination is described e.g. by the following equation (25):

$$C_a = A \times e^{-k_a \times t} + B \times e^{k_b \times t} \qquad \text{[equation 4]}$$

A and B represent the size of the compartments (in percent of overall tissue residue), where A is the compartment with rapid loss of substance, and B the compartment with slow loss of test substance. The sum of A and B equals 100% of the whole animal compartment volume at steady state. k_a and k_b represent the corresponding elimination constants [d^{-1}]. If the two compartment model is fitted to the depuration data, the uptake rate constant k_s may be determined as follows (53)(54):

$$k_s = \frac{(A \times k_a + B \times k_b) \times BAF}{A+B} \qquad \text{[equation 5]}$$

Nevertheless, these model equations should be used with caution, especially when changes in the test substance's bioavailability occur during the test (42).

As an alternative to the model equations described above, the kinetics (k_s and k_e) may also be calculated in one run by applying the first order kinetics model to all data from both the uptake and elimination phase together. For a description of a method that may allow for such a combined calculation of uptake and elimination rate constants, references (55), (56) and (57) may be consulted.

The Non-Eliminated Residues (NER) should be calculated as a secondary endpoint by multiplying the ratio of the average concentration in the worms (C_a) on day 10 of the elimination phase and the average concentration in the worms (C_a) at steady state (day 28 of uptake phase) by 100:

$$\boldsymbol{NER_{10d}}[\%] = \frac{\boldsymbol{C_a\ at\ end\ of\ elimination\ (average)} \times \mathbf{100}}{\boldsymbol{C_a at\ steady\ state\ (average)}}$$

APPENDIX 3

EXAMPLE OF A SAMPLING SCHEDULE FOR A 28-DAY BIOACCUMULATION TEST

a) Uptake phase (including a 4 d- equilibration phase)

Day	Activities
-6	Preparation of peat suspension for sediment; conditioning of the suspension for 48 h;
-4	Spiking of the sediment or sediment fraction; mixing of all sediment constituents; removing sediment samples of treated and solvent control sediment for determination of test substance concentration; addition of overlying water; incubation at test conditions (equilibration phase);
-3/-2	Separation of the test organisms from the culture for acclimatisation;
0	Measurement of water quality (see paragraph 52); removing replicates for taking samples of water and sediment for determination of test substance concentration; randomised distribution of the worms to the test chambers; retaining of sufficient sub-samples of worms for determination of analytical background values; controlling air supply, if closed test system is used;
1	Remove replicates for sampling; controlling air supply, worm behaviour, water quality (see paragraph 56); taking water, sediment and worm samples for determination of test substance concentration;
2	Controlling air supply, worm behaviour and temperature;
3	Same as day 1;
4 - 6	Same as day 2;
7	Same as day 1; compensate evaporated water if necessary;
8 - 13	Same as day 2;
14	Same as day 1; compensate evaporated water if necessary;
15 - 20	Same as day 2;
21	Same as day 1; compensate evaporated water if necessary;
22 - 27	Same as day 2;
28	Same as day 1; measurement of water quality (see paragraph 52); end of uptake phase; retaining of sufficient subsamples of worms for determination of analytical background values, wet and dry weight, and lipid content; transfer worms from remaining exposed replicates to vessels containing clean sediment for elimination phase (no gut-purging); sampling of water, sediment and worms from solvent controls; sampling of trapping solutions, if installed.
	Pre-exposure activities (equilibration phase) should be scheduled taking into account the properties of the test substance. If required, conditioning of the prepared sediment under overlying water at 20 ± 2°C for 7 days; in this case, earlier preparation of the sediment!
	Activities described for day 2 should be performed daily (at least on workdays).

b) Elimination phase

Day	Activities
-6	Preparation of peat suspension for sediment; conditioning of the suspension for 48 h;
-4	Mixing of all sediment constituents; removing sediment samples of treated and solvent control sediment for determination of test substance concentration; addition of overlying water; incubation at test conditions;
0 (day 28 of uptake phase)	Measurement of water quality (see paragraph 52); transfer worms from remaining exposed replicates to vessels containing clean sediment; after **4 - 6 h** removing replicates for taking samples of water, sediment and worms for determination of test substance concentration; randomised distribution of the worms to the test chambers;
1	Remove replicates for sampling; controlling air supply, worm behaviour, water quality (see paragraph 52); taking water, sediment and worm samples for determination of test substance concentration;
2	Controlling air supply, worm behaviour and temperature;
3	Same as day 1;
4	Same as day 2;
5	Same as day 1;
6	Same as day 2;
7	Same as day 1; compensate evaporated water if necessary;
8 - 9	Same as day 2;
10	Same as day 1; end of elimination phase; measurement of water quality (see paragraph 52); sampling of water, sediment and worms from solvent controls; sampling of trapping solutions, if installed.
	Preparation of the sediment prior to start of elimination phase should be done in the same manner as before the uptake phase.
	Activities described for day 2 should be performed daily (at least on workdays).

APPENDIX 4

SOME PHYSICAL-CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

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

COMPOSITION OF THE RECOMMENDED RECONSTITUTED WATER

(a) Calcium chloride solution

Dissolve 11.76 g $CaCl_2 \cdot 2H_2O$ in deionised water; make up to 1 l with deionised water

(b) Magnesium sulphate solution

Dissolve 4.93 g $MgSO_4 \cdot 7H_2O$ in deionised water; make up to 1 l with deionised water

(c) Sodium bicarbonate solution

Dissolve 2.59 g $NaHCO_3$ in deionised water; make up to 1 l with deionised water

(d) Potassium chloride solution

Dissolve 0.23 g KCl in deionised water; make up to 1 l with deionised water

All chemicals must be of analytical grade.

The conductivity of the distilled or deionised water should not exceed 10 μScm^{-1}.

25 ml each of solutions (a) to (d) are mixed and the total volume made up to 1 l with deionised water. The sum of the calcium and magnesium ions in this solution is 2.5 mmol/l.

The proportion Ca:Mg ions is 4:1 and Na:K ions 10:1. The acid capacity $K_{S4.3}$ of this solution is 0.8 mmol/l.

Aerate the dilution water until oxygen saturation is achieved, then store it for approximately two days without further aeration before use.

The pH of an acceptable dilution water should be in the range of 6 - 9.

APPENDIX 5

ARTIFICIAL SEDIMENT - PREPARATION AND STORAGE RECOMMENDATIONS

In contrast to the requirements in test method C.8 (40) the peat content of the artificial sediment is recommended to be 2% instead of 10% of dry weight, in order to correspond to a low to moderate organic content of natural sediments (58).

Percentage of dry constituents of the artificial sediment:

Constituent	**Characteristics**	**% of dry sediment**
Peat	Sphagnum moss peat, degree of decomposition: "medium", air dried, no visible plant remains, finely ground (particle size ≤ 0.5 mm)	2 ± 0.5
Quartz sand	Grain size: ≤ 2 mm, but > 50% of the particles should be in the range of 50-200 µm	76
Kaolinite clay	Kaolinite content ≥ 30%	22 ± 1
Food source	*Folia urticae*, powdered leaves of *Urtica sp.* (stinging nettle), finely ground (particle size ≤ 0.5 mm), or a mixture of powdered leaves of *Urtica sp.* with alpha-cellulose (1 : 1); in accordance with pharmacy standards, for human consumption; in addition to dry sediment	0.4 - 0.5%
Calcium carbonate	$CaCO_3$, pulverised, chemically pure, in addition to dry sediment	0.05 - 1
Deionised Water	Conductivity ≤ 10 µS/cm, in addition to dry sediment	30 - 50

If elevated ammonia concentrations are expected, e.g. if the test substance is known to inhibit the nitrification, it may be useful to replace 50% of the nitrogen-rich urtica powder by cellulose (e.g., α-Cellulose powder, chemically pure, particle size ≤ 0.5 mm).

Preparation

The peat is air-dried and ground to a fine powder (grain size ≤ 0.5 mm, no visible plant remains). A suspension of the required amount of peat powder is prepared using a portion of the deionised water to be added to the dry sediment (a water volume of 11.5 x dry weight of peat has been found useful to produce a stirrable peat slurry (8)) using a high-performance homogenising device.

The pH of this suspension is adjusted to 5.5 ± 0.5 with $CaCO_3$. The suspension is conditioned for at least two days with gentle stirring at 20 ± 2 °C, to stabilise pH and establish a stable microbial component. The pH is measured again and is adjusted to 6.0 ± 0.5 with $CaCO_3$ if necessary. Then all of the suspension is mixed with the other dry constituents, taking into account any portion used for spiking. The remaining deionised water is added to obtain a homogeneous sediment. The pH is measured again and is adjusted to 6.5 to 7.5 with $CaCO_3$ if necessary. However, if ammonia development is expected, it may be useful to keep the pH of the sediment below 7.0 (e.g. between 6.0 and 6.5). Samples of the sediment are taken to determine the dry weight and the organic carbon content. If ammonia development is expected, the artificial sediment may be conditioned for seven days under the same conditions which prevail in the subsequent test (e.g. sediment-water ratio 1 : 4, height of sediment layer as in test vessels) before it is spiked with the test substance, i.e. it should be topped with water, which should be aerated. At the end of the conditioning period, the overlying water should be removed and discarded. Samples of the sediment are taken to determine dry weight and total organic carbon content (e.g. 3 samples).

Thereafter, the spiked quartz sand is mixed with the sediment for each treatment level, the sediment is distributed to the replicate test vessels, and topped with the test water (e.g. sediment-water ratio 1 : 4, height of sediment layer as in test vessels). The vessels are then incubated at the same conditions which prevail in the subsequent test. This is where the equilibration period starts. The overlying water should be aerated.

The chosen food source should be added prior to or during spiking the sediment with the test substance. It can be mixed initially with the peat suspension (see above). However, excessive degradation of the food source prior to addition of the test organisms - e.g. in case of long equilibration period - can be avoided by keeping the time period between food addition and start of exposure as short as possible. In order to ensure that the food is in sufficient contact with the test substance, the food source should be mixed with the sediment not later than on the day the test substance is spiked to the sediment. Exceptions may be made where the length of the equilibration period leads to excessive microbial degradation of the food before the test organisms are added. Samples of the sediment are taken to determine dry weight and total organic carbon (e.g. 3 samples of spiked or control sediment).

The dry weight of the components (peat, sand, kaolin) should be reported in g and in per cent of total dry weight.

The volume of water to be added to the dry components during preparation of the sediment should also be reported in per cent of total dry weight (e.g. 100% dry weight + 46% water means 1000 g d.w. receive a total of 460 ml water, which results in 1460 g wet sediment).

Storage

The dry constituents of the artificial sediment may be stored in a dry, cool place at room temperature. The prepared, wet sediment may be stored (for further use in the culture only) at 4 ± 2°C in the dark for a period of 2 to 4 weeks from the day of preparation (8).

Sediment spiked with the test substance should be used immediately unless there is information indicating that the particular sediment can be stored without affecting the toxicity and bioavailability of the test substance. Samples of spiked sediment may be stored under the conditions recommended for the particular test substance until analysis.

APPENDIX 6

OLIGOCHAETES SPECIES RECOMMENDED FOR BIOACCUMULATION TESTING

Tubifex tubifex (MÜLLER),Tubificidae, Oligochaeta

The tubificid oligochaete (Tubificidae, Oligochaeta) *Tubifex tubifex* (Müller) lives in freshwater sediments in tubes which are lined with mucus. In these tubes the worms dwell head down, ingesting sediment particles utilising the associated microorganisms and organic debris. The posterior portion usually undulates in the overlying water for respiration purposes. Although this species inhabits a wide range of sediment types all over the northern hemisphere, *Tubifex tubifex* prefers relatively fine grain sizes (59). The suitability of this species for ecotoxicological testing is described for example in (8)(29)(31)(39)(60)(62)(63).

Culture methods

In order to have a sufficient number of *Tubifex tubifex* for conducting bioaccumulation tests the worms have to be kept in permanent laboratory culture. A system consisting of artificial sediment based on the artificial soil according to Test Method C.8 (40) and reconstituted water according to test method C.1 is recommended for *T. tubifex* culture (8).

Glass or stainless steel containers with a height of 12 to 20 cm can be used as culture vessels. Each culture container is loaded with a layer of wet artificial sediment prepared as described in Appendix 5. The depth of the sediment layer should allow for natural burrowing behaviour of the worms (2 cm minimum depth for *T. tubifex*). Reconstituted water is added to the system. Care should be taken to minimise disturbing the sediment. The water body is gently aerated (e.g. 2 bubbles per second with 0.45 µm-filtered air) via a pasteur pipette positioned 2 cm above the sediment surface. The recommended culture temperature is 20 ± 2°C.

The worms are added to the culture system with a maximum loading of 20,000 individuals/m^2 sediment surface. A higher loading may cause a reduction in growth and reproduction rates (43).

In artificial sediment cultures, the worms have to be fed. A diet consisting of finely ground fish food, e.g. TetraMin® can serve as additional nutrition (8); Klerks 1994, personal communication. The feeding rates should allow for sufficient growth and reproduction and should keep build-up of ammonia and fungal growth in the culture at a minimum. Food may be administered twice a week (e.g. 0.6 - 0.8 mg per cm^2 of sediment surface). Practical experience has shown that application of food suspended and homogenised in deionised water may facilitate homogeneous food distribution on the sediment surface in the culture containers.

To avoid accumulation of ammonia, the overlying water should be exchanged using a flow-through system, or, at least once a week, manually. Sediment should be changed every three months in the stock cultures.

Sampling of worms from the culture can be done by sieving the culture sediment through a 1 mm sieve if only adults are required. For retaining cocoons a 0.5 mm mesh, and for juvenile worms a 0.25 mm sieve is suitable. The sieves can be placed into reconstituted water after the sediment has passed through. The worms leave the mesh and can then be picked from the water using a soft steel forceps or a pipette with fire-polished edges.

Only intact and clearly identified specimens of *Tubifex tubifex* (e.g. (64)) are used to start a test or new cultures. Diseased or injured worms as well as cocoons infested with fungal hyphae have to be discarded.

A synchronised culture can provide worms of a specified age in suitable intervals when desired. New culture vessels are set up in the chosen intervals (e.g. every two weeks), starting with animals of a certain age (e.g. cocoons). At the culture conditions described here the worms are adult after 8 - 10 weeks. The cultures can be harvested, when the worms have laid new cocoons, e.g. after ten weeks. The sampled adults can be used for tests, and new cultures can be started with the cocoons.

***Lumbriculus variegatus* (MÜLLER), Lumbriculidae, Oligochaeta**

Lumbriculus variegatus (Lumbriculidae, Oligochaeta) is also an inhabitant of freshwater sediments worldwide and is widely used in ecotoxicological testing. Information on the biology, culture conditions, and sensitivity of the species can be obtained from (1)(6)(9)(36). *Lumbriculus variegatus* can also be cultured in the artificial sediment recommended for *T. tubifex* according to (8) within certain limitations. Since, in nature *L. variegatus* prefers more coarse sediments than *T. tubifex (59)*, laboratory cultures with the artificial sediment used for *T. tubifex* may cease after 4 to 6 months. Practical experience has shown that *L. variegatus* can be held in a sandy substratum (e.g. quartz sand, fine gravel) in a flow-through system using fish food as nutritional source over several years without renewing the substratum. A major advantage of *L. variegatus* over other aquatic oligochaete species is its quick reproduction, resulting in rapidly increasing biomass in laboratory-cultured populations (1)(6)(9)(10).

Culture methods

Culture conditions for *Lumbriculus variegatus* are outlined in detail in Phipps et al. (1993) (10), Brunson et al. (1998) (28), ASTM (2000) (1), U.S. EPA (2000) (6). A short summary of these conditions is given below.

The worms can be cultured in large aquaria (57 - 80 l) at 23°C with a 16L:8D photoperiod (100 - 1000 lux) using daily renewed natural water (45 - 50 l per aquarium). The substrate is prepared by cutting unbleached brown paper towels into strips, which may then be blended with culture water for a few seconds to result in small pieces of paper substrate. This substrate can then directly be used in the *Lumbriculus* culture aquaria by covering the bottom area of the tank, or be stored frozen in deionised water for later use. New substrate in the tank will generally last for about two months.

Each worm culture is started with 500 - 1,000 worms, and fed a 10 ml suspension containing 6 g of trout starter food 3 times per week under renewal or flow-through

conditions. Static or semi-static cultures should receive lower feeding rates to prevent bacterial and fungal growth. Food and paper substrate should be analysed for the substances to be used in bioaccumulation tests.

Under these conditions the number of individuals in the culture generally doubles in about 10 to 14 d.

Lumbriculus variegatus can be removed from the cultures e.g. by transferring substrate with a fine mesh net, or organisms using a fire polished wide mouth (about 5 mm diameter) glass pipette, to a separate beaker. If substrate is co-transferred to this beaker, the beaker containing worms and substrate is left overnight under flow-through conditions, which will remove the substrate from the beaker, while the worms remain at the bottom of the vessel. They can then be introduced to newly prepared culture tanks, or processed further for the test as outlined in (1) and (6). Injuries or autotomy in the worms should be prevented, e.g. by using pipettes with fire polished edges, or stainless steel picks for handling these worms.

An issue to be regarded critically when using *L. variegatus* in sediment bioaccumulation tests is its reproduction mode (architomy followed by morphallaxis). This asexual reproduction results in two fragments, which do not feed for a certain period until the head or tail part is regenerated (e.g. (36)(37)). This means that in *L. variegatus* sediment and contaminant uptake via ingestion may not take place continuously as in tubificids, which do not reproduce by fragmentation.

Therefore, a synchronisation should be performed to minimise uncontrolled reproduction and regeneration, and subsequent high variation in test results. Such variation can occur, when some individuals, which have fragmented and therefore do not feed for a certain time period, are less exposed to the test substance than other individuals, which do not fragment during the test, e.g. (38). 10 to 14 days before the start of exposure, the worms should be artificially fragmented (synchronisation) (65). Large worms should be used, which preferably do not show signs of recent fragmentation. These worms can be placed onto a glass slide in a drop of culture water, and dissected in the median body region with a scalpel. Care should be taken that the posterior ends are of similar size. The posterior ends should then be left to regenerate new heads in a culture vessel containing the same substrate as used in the culture and reconstituted water until the start of exposure. Regeneration of new heads is indicated when the synchronised worms are burrowing in the substrate (presence of regenerated heads may be confirmed by inspecting a representative subsample under a binocular microscope). The test organisms are thereafter expected to be in a similar physiological state. This means, that when regeneration by morphallaxis occurs in synchronised worms during the test, virtually all animals are expected to be equally exposed to the spiked sediment. Feeding of the synchronised worms should be done as soon as the worms are starting to burrow in the substrate, or 7 d after dissection. The feeding regimen should be comparable to the regular cultures, but it may be advisable to feed the synchronised worms with the same food source as is to be used in the test. The worms should be held at test temperature, at 20 ± 2°C. After regenerating, intact complete worms of similar size, which are actively swimming or crawling upon a gentle mechanical stimulus, should be used for the test. Injuries or autotomy in the worms should be prevented, e.g. by using pipettes with fire polished edges, or stainless steel picks for handling these worms.

When using *Lumbriculus variegatus* in the test, due to the specific reproduction mode of this species, an increase of the number of worms should occur during the test, if conditions are appropriate (6). A lack of reproduction in a bioaccumulation test with *L. variegatus* should be recorded, and considered when interpreting the test results.

***Branchiura sowerbyi* (BEDDARD), Tubificidae, Oligochaeta (not validated in ring test)**

Branchiura sowerbyi inhabits a variety of sediment types of reservoirs, lakes, ponds and rivers, originally in tropical areas. They can be also found in warm water bodies of the northern hemisphere. However, they are more abundant in mud-clay sediments with high organic matter content. Furthermore, the worms are living in the sediment layer. Even the posterior end of the worms is usually burrowed. This species is easily identified from the gill filaments on their posterior part. The adults can reach a length of 9 - 11 cm and a wet weight of 40-50 mg. The worms have a high rate of reproduction, show population doubling times of less than 2 weeks and under the conditions of temperature and feeding described below (Aston et al., 1982, (65)). *B. sowerbyi* has been used both in toxicity and bioaccumulation studies (Marchese & Brinkhurst 1996, (31) Roghair et al. 1996, (67) respectively).

Culture methods

A summary of culture conditions for *Branchiura sowerbyi* is given below (provided by Mercedes R. Marchese, INALI, Argentina, and Carla J. Roghair, RIVM, The Netherlands).

No single technique for culturing the test organisms is required. The organisms can be cultured using uncontaminated, natural sediment (31). Practical experience showed that a medium consisting of natural sediment and sand improves the condition of the worms compared to pure natural sediment (32)(67). 3 L-beakers containing 1 500 ml sediment/water medium, consisting of 375 ml of natural uncontaminated sediment (about 10 % Total Organic Carbon; about 17% of the particles ≤ 63 µm), 375 ml of clean sand (M32), and 750 ml of reconstituted or dechlorinated tap water can be used for the culture (31)(32)(67). Paper towels also can be used as a substrate for culturing, but population growth is lower than in natural sediment. In semi-static systems the water layer in the beaker is slowly aerated, and the overlying water should be renewed weekly.

Each beaker contains 25 young worms to start with. After two months the large worms are picked out of the sediment with a pair of tweezers and are put in a new beaker with freshly made sediment/water medium. The old beaker also contains cocoons and young worms. Up to 400 young worms per beaker can be harvested in this way. Adults worms can be used for reproduction for at least one year.

The cultures should be maintained at a temperature of 21 to 25°C. Variation of temperature should be kept below ± 2°C. The time required for embryonic development from an egg being laid until the young leaves the cocoon is approximately three weeks at 25°C. The egg production obtained per surviving worm in *B. sowerbyi* was found to range from 6.36 (31) to 11.2 (30) in mud at 25°C. The number of eggs per cocoon ranges from 1.8 to 2.8 (66)(69) or up to 8 (68).

Dissolved oxygen, water hardness, temperature, and pH should be measured weekly.

Fish food (e.g. TetraMin®) can be added as suspension two or three times per week ad libitum. The worms can also be fed with thawed lettuce *ad libitum*.

A major advantage of this species is the high individual biomass (up to 40 - 50 mg wet weight per individual). Therefore this species may be used for testing bioaccumulation of non-radiolabelled test substances. It can be exposed in the systems used for *T. tubifex* or *L. variegatus* with a single individual per replicate (11). Replication, however, should then be increased, unless larger test chambers are used (11). Also, the validity criterion related to burrowing behaviour needs to be adjusted for this species.

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Chapter A.4, vapour pressure
Chapter A.5, Surface tension
Chapter A.6, Water solubility
Chapter A.8, Partition coefficient, shake flask method
Chapter A.24, Partition coefficient, HPLC method
Chapter C.7, degradation — abiotic degradation: hydrolysis as a function of pH
Chapter C.4 A-F Determination of ready biodegradability
Chapter C.19, Estimation of the adsorption coefficient (K_{oc}) on soil and on sewage sludge using high performance liquid chromatography (HPLC)
Chapter C.29, Ready biodegradability CO_2 in sealed vessels

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