Section 2
Effects on Biotic Systems

Test Guideline No. 219

Sediment-Water Chironomid Toxicity Using Spiked Water

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OECD Guidelines for the Testing of Chemicals

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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Sediment-water Chironomid Toxicity Test Using Spiked Water

INTRODUCTION

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

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

3. Chemicals that need to be tested towards sediment-dwelling organisms usually persist in this compartment over long time periods. The sediment-dwelling organisms may be exposed via a number of routes (i.e. waterborne, sediment and dietary exposure). The relative importance of each exposure route, and the time taken for each to contribute to the overall toxic effects, is dependent on the physical-chemical properties of the chemical concerned. For strongly adsorbing chemicals (e.g. with log K_{ow} > 5) 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 is expected to be considered. In order to take all potential routes of exposure into account the focus of this Guideline is on long-term exposure. The test duration is in the range of 20 - 28 days for *C. riparius* and *C. yoshimatsui*, and 28 - 65 days for *C. dilutus*. If short-term data are required for a specific purpose, for example to investigate the effects of unstable chemical, additional replicates may be removed after a 10-day period.

4. The measured endpoints are based on the following biological variables: the number of emerged males, the number of emerged females (i.e. thus also the total number of adults emerged) as well as the time to emergence. Measurements of larval survival and growth should only be made after a 10-day period if additional short-term data are required, using additional replicates as appropriate.

5. The use of formulated sediment (also called reconstituted, artificial or synthetic sediment) is recommended. Formulated sediment has several advantages over natural sediments:

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

6. Definitions used are given in Annex 1.

PRINCIPLE OF THE TEST

7. First instar chironomid larvae are exposed to a concentration range of the test chemical in sediment-water systems. The test starts by placing first instar larvae into the test beakers containing the sediment-water system and subsequently spiking the test chemical into the water. Chironomid emergence and development rate is measured at the end of the test. Larval survival and weight may also be measured after 10 days if required (using additional replicates as appropriate). These data are analysed either by using a regression model in order to estimate the concentration that would cause x % reduction in emergence, larvae survival or growth (e.g. EC_{10}, EC_{50}, etc.), or by using statistical hypothesis testing to determine a NOEC/LOEC. The latter requires comparison of effect values with control values using statistical tests. Depending on the requirements of the respective regulatory framework, the test design should be optimised to estimate an ECx endpoint and/ or to determine a NOEC/LOEC (see section TEST DESIGN).

INFORMATION ON THE TEST CHEMICAL

8. The water solubility of the test chemical, its vapour pressure, measured or calculated octanol-water partition coefficient and 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 limits of detection and quantification should be available. Useful information includes the structural formula andpurity of the test chemical. Chemical fate of the test chemical (e.g. dissipation, abiotic and biotic degradation, etc.) also is useful information. Further guidance for testing chemicals with physico-chemical properties that make them difficult to perform the test is provided in (12).

REFERENCE SUBSTANCES

9. The testing of a reference chemical should be carried out regularly to assure that the test protocol and test conditions are reliable. Examples of reference toxicants used successfully in ring-tests and validation studies are: lindane, trifluralin, pentachlorophenol, cadmium chloride and potassium chloride. (1)(2)(5)(6)(13).

VALIDITY OF THE TEST

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

- the emergence in each control replicate should be at least 70 % at the end of the test (1) (6); please note that data should be reported for each replicate (see paragraph 54);

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- *C. riparius* and *C. yoshimatsui* emergence to adults from control vessels should occur between 12 and 23 days after their insertion into the vessels; for *C. dilutus*, a period of 20 to 65 days is necessary;
- throughout the test period, the oxygen concentration should be at least 60 % of the air saturation value (ASV) at the temperature used, and the pH of overlying water should be between 6 to 9 range in all test vessels;
- throughout the test period, the water temperature should not differ by more than ± 1.0 °C.

DESCRIPTION OF THE METHOD

Test Vessels

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

Test species

12. The species to be used in the test is preferably *C. riparius. C. dilutus* is also suitable but more difficult to handle and requires a longer test period. *C. yoshimatsui* may also be used. Details of culture methods are given in Annex 2 for *C. riparius*. Information on culture conditions is also available for other species, i.e. *C. dilutus* (4)(14) and *C. yoshimatsui* (11). Identification of species should be confirmed before testing but is not required prior to every test if organisms come from an in-house culture.

Sediment

13. Formulated sediment should preferably be used. However, if natural sediment is used, it should be characterised (at least pH and organic carbon content should be measured; in addition, it is recommended to determine other parameters such as C/N ratio and granulometry), and it should be free from any contamination and other organisms that might compete with, or consume the chironomids. It is also recommended that, before it is used in a chironomid toxicity test, the sediment be conditioned for seven days under the same conditions which prevail in the subsequent test.

The following formulated sediment, based on the artificial soil used in Guideline 222 (15), is recommended for use in this test (1)(16)(17):

1. 4 - 5 % (dry weight) peat (e.g. Sphagnum): 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.
2. 20 % (dry weight) kaolin clay (kaolinite content preferably above 30 %).

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3. 75 - 76 % (dry weight) quartz sand (fine sand should predominate with more than 50 % of the particles between 50 and 200 μm).
4. Deionised water is added to obtain a sediment with a water content in a range of 30-50 %.
5. Calcium carbonate of chemically pure quality ($CaCO_3$) is added adjust the pH of thefinal mixture of the sediment to 7.0 ± 0.5.
6. 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, etc.). An example for the preparation of the formulated sediment is described in Annex 3. Mixing of dry constituents is also acceptable if it is demonstrated that after addition of overlying water a separation of sediment constituents (e.g. floating of peat particles) does not occur, and that the peat or the sediment is sufficiently conditioned.

15. Sediment type and sediment properties can substantially affect the fate and bioavailability of the test chemical, and therefore uptake by the test organism and subsequent toxicity. If natural sediment is used, it is necessary that sediment characteristics that may have an influence on bioavailability and therefore toxicity are reported. Total organic carbon usually provides the main binding phase for many types of chemicals and therefore its concentration in natural sediment should be measured so that normalisation of toxicity data to organic carbon can be done (18)(19) (see paragraph 45).

Water

16. Reconstituted water should preferably be used. However, any water which conforms to the chemical characteristics of acceptable dilution water as listed in Annexes 2 and 4 is suitable as test water. Any suitable water, natural water (surface or ground water), reconstituted water (see Annex 2) or dechlorinated tap water are acceptable as culturing water and test water if chironomids will survive in it for the duration of the culturing and testing without showing signs of stress (e.g. .unusual behaviour). 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 whole study. The water quality characteristics listed in Annex 4 should be measured at least twice a year if natural or tap water is used or when it is suspected that these characteristics may have changed significantly. If using natural water, special care should be taken considering the characteristics mentioned.

Stock solutions - Spiked water

17. 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 medium. If the test chemical is poorly soluble in water, the use of solvents or dispersants may not be avoidable, i.e. it is needed to produce a suitably concentrated stock solution. In such case, the OECD Guidance Document No. 23 (12) should be consulted. Examples of suitable solvents are acetone, ethanol, methanol, ethylene glycol monoethyl ether, ethylene glycol dimethyl ether, dimethylformamide and triethylene glycol. Dispersants which may be used are Cremophor RH40, Tween 80, methylcellulose 0.01 % and HCO-40. The solubilising agent concentration in the

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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 nor visible adverse effect on the chironomid larvae as revealed by a solvent-only control. However, every effort should be made to avoid the use of such materials.

TEST DESIGN

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

Design for analysis by regression

19. The effect concentration (e.g. EC_{10}, EC_{50}) and the concentration range, over which the effect of the test chemical is of interest, should be spanned by the concentrations included in the test. Generally, the accuracy and especially validity, with which estimates of effect concentrations (ECx) can be made, is improved when the effect concentration is within the range of concentrations tested. Extrapolating outside the concentration range tested should be avoided. A preliminary range-finding test is helpful for selecting the range of concentrations to be used (see paragraph 28).

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

Design for estimation of a NOEC/LOEC

21. If the LOEC/NOEC are to be estimated, five test concentrations with at least four replicates should be used and the factor between concentrations should not be greater than two. The number of replicates should be sufficient to ensure adequate statistical power to detect a 20 % difference from the control at the 5% level of significance (p = 0.05). With the development rate, an Analysis of Variance (ANOVA) is usually appropriate, such as Dunnett-test and Williams-test (20)(21)(22)(23). For the emergence data, the Cochran-Armitage, Fisher's exact (with Bonferroni correction), or Mantel-Haentzal tests may be used. Alternatively, the CPFISH (closure principle and Fisher–Freeman–Halton) test may be used since this test has a high statistical power (24).

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Limit test

22. A limit test may be performed (one test concentration and control) if no effects were seen in the preliminary range-finding test. The purpose of the limit test is to perform a test at a concentration sufficiently high to enable decision makers to exclude possible toxic effects of the chemical, and the limit is set at a concentration which is not expected to appear in any situation. No suggestion for a recommended concentration can be made in this guideline; this is left to the regulators' judgement. Usually, at least six replicates for both the treatment and control are necessary. Adequate statistical power to detect a 20 % difference from the control at the 5 % level of significance (p = 0.05) should be demonstrated. With metric response (development rate and weight), the t-test is a suitable statistical method if data meet the requirements ofthis test (normality, homogeneous variances). The unequal-variance t-test or a non-parametric test, such as the Wilcoxon-Mann-Whithey test may be used, if these requirements are not fulfilled. With the emergence ratio, the Fisher exact test is appropriate.

PROCEDURE

Conditions of exposure

Preparation of spiked water-sediment system

23. Appropriate amounts of formulated sediment (see paragraphs 13-14 and Annex 3) are added inthe test vessels to form a layer of 1.5 ± 0.25 cm. The weight of the sediment added to each test chamber (or at least the test chambers being used for analytical confirmation) should be recorded. This information is needed to calculate the mass balance (see paragraph 43). Water is added to a depth of 6 cm (see paragraph 16). The ratio of the depth of the sediment layer and the depth of the water should be 1:4 and the sediment layer should not be deeper than 1.5 ± 0.25 cm. The sediment-water system should be left under gentle aeration for stabilisation for seven days prior to addition of test organisms (see paragraph 14 and Annex 3). To avoid separation of sediment ingredients and re-suspension of fine material during addition of test water in the water column, the sediment can be covered with a disc (made of e.g. stainless steel or PTFE) while water is poured onto it, and the disc is removed immediately afterwards. Other devices may also be appropriate.

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

Addition of test organisms

25. Four to five days before adding the test organisms to the test vessels, egg masses should be taken from the cultures and placed in small vessels in culture medium. Aged medium from the stock culture or freshly prepared medium may be used. If the latter is used, a small amount of food, for example green algae and/or a few droplets of filtrate from a finely ground suspension of flaked fish food should be added to the culture medium (see Annex 2). Only freshly laid egg masses should be used. Normally, the larvae begin to hatch a couple of days after the eggs are laid (2 to 3 days for *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 to 8 days duration. First instar larvae ((2-3 days post-hatch for *C. riparius*; 1-4 days post-hatch for *C. dilutus* and *C. yoshimatsui*)) should be used in the test. In case of uncertainties on the instar of midges, it can possibly be checked using head capsule width (6).

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26. Twenty first instar larvae are allocated randomly to each test vessel containing the sediment and water, using a blunt pipette. Gentle aeration of the water has to be stopped while adding the larvae to test vessels and remain so for another e.g. 4 hours after addition of larvae (see paragraphs 25 and 33). According to the main test design used (see paragraphs 20 and 21), the number of larvae used per concentration is at least 60 for the EC point estimation and 80 for determination of NOEC.

27. Twenty-four hours after adding the larvae, aeration is stopped again and the test chemical is spiked into the overlying water column. Small volumes of 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. When spiking is finished, aeration is re-started and samples for analytical determination of test chemical concentrations are taken, preferably one hour after application of the test chemical.

Test concentrations

28. A range-finding test may be helpful to determine the range of concentrations for the definitive test. For this purpose, a series of widely spaced concentrations of the test chemical are used. The chironomids are exposed to each concentration of the test chemical for a period which allows estimation of appropriate test concentrations, and no replicates are required. The larval density per vessel should be the same as for the definitive test. For the definitive test, at least five concentrations should be used and selected as described in paragraphs 19 to 21.

Controls

29. Control vessels without any test chemical but including sediment should be included in the test with the appropriate number of replicates (see paragraphs 20-21). If a solvent has been used for application of test chemical (see paragraph 17), a solvent control (water spiked with solvent only) but including sediment should be performed in addition to the usual control (or negative control). It should correspond to the highest solvent concentration used in the test treatments and generally not exceed 0.01%.

Test system

30. Static systems are used. If evaporation occurs during the test, the volume of overlying water should be regularly adjusted by adding distilled or deionised water. Semi-static or flow-through systems with intermittent or continuous renewal of overlying water might be used in exceptional cases as for instance if water quality specifications become inappropriate for the test organism or affect chemical equilibrium (e.g. dissolved oxygen levels or the concentration of excretory products exceed the respective appropriate limits or minerals leach from sediment and affect pH and/or water hardness). However, other methods for ameliorating the quality of overlying water, such as aeration, will normally suffice and be preferable.

Food

31. It is necessary to feed the larvae, preferably daily or at least three times per week. Fish-food (a suspension in water or finely ground food, for example TetraMin® or TetraPhyll®; see details in Annex 2) in the amount of 0.25-0.5 mg (0.35-0.5 mg for *C. yoshimatsui*) per larvae per day seems adequate for young larvae for the first 10 days. Slightly more food may be necessary for older larvae: 0.5-1 mg per larvae per day should be

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sufficient for the rest of the test. Midge do not feed when they reach the pupal stage, so the feeding regime may be adapted to the number of larvae that have pupated and/or emerged to avoid low dissolved oxygen levels. The food ration should be reduced in all treatments and control if fungal growth is seen or if mortality is observed in controls. If fungal development cannot be stopped the test is to be repeated. When testing strongly adsorbing chemicals (e.g. with log K_{ow} > 5), or chemicals covalently binding to sediment, the amount of food necessary to ensure survival and natural growth of the organisms should be added to the formulated sediment before the stabilisation period. For this, plant material should be used instead of fish food, for example the addition of 0.5% (dry weight) finely ground leaves of stinging nettle (*Urtica dioeca*), mulberry (*Morus alba*), white clover (*Triflorium repens*), spinach (*Spinacia oleracea*) or of other plant material (dried and milled wheat grass or alpha-cellulose) may be used (25). Alternatively, field-collected sediment with sufficiently high nutritional content for the total test period could be used instead of standard OECD sediment which has lower nutritional value as far as chironomids survive in it for the duration of the testing without showing signs of stress (e.g. emigration of larvae towards water column, unusual behaviour).

Incubation conditions

32. Gentle aeration of the overlying water in test vessels is supplied preferably e.g. 4 hours afteraddition of the larvae and is continued throughout the test (care should be taken that dissolved oxygen concentration does not fall below 60 % of ASV). Aeration is provided through a glass Pasteur pipette fixed 2-3 cm above the sediment layer (i.e. one or few bubbles/sec). When testing volatile chemicals, consideration may be given not to aerate the sediment-water system.

33. The test is conducted at a constant temperature of 20 °C (± 2 °C) for *C. riparius*. For *C. dilutus* and *C. yoshimatsui*, recommended temperatures are of 23 °C and 25 °C (± 2 °C), respectively. If the test is conducted in an isothermal room, the room temperature should be verified at appropriate time intervals (at least once daily). A 16 hours photoperiod is used and the light intensity should be 500 to 1000 lx.

Exposure duration

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

Observations

Emergence

35. The number of fully emerged males, the number of fully emerged females as well as the total number of emerged midges (males and females) are recorded daily. Males are easily identified by their plumose antennae. The respective development rates are calculated according to paragraphs 52 and 53.

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

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Growth and survival

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

Analytical measurements

Concentration of the test chemical

38. For the analytical determination of the test chemical concentration, samples of the overlying water, the pore water and the sediment should be taken for analysis at least at the start (preferably one hour after application of test chemical) and at the end of the test, at least at the highest concentration and a lower one, but preferably at all tested concentrations and from at least one vessel per treatment. These determinations of test chemical concentration inform on the behaviour/partitioning of the tested chemical in the water-sediment system. Measurements in sediment 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).

39. If the analysis needs large sampleswhich cannot be taken from test vessels without influencing the test system (e.g. because samples are taken at different times of the test), analytical determinations should be performed on samples from additional test vessels treated in the same way (including the presence of test organisms) but not used for biological observations.

40. Centrifugation at, for example, 10,000 g and 4 °C for 30 min. is the recommended procedure to isolate 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 size is too small.

Physical-chemical parameters

41. The pH, dissolved oxygen and temperature should be measured at least in one vessel of each concentration level and one vessel of control(s) at the start of exposure and once per week 2 and 3 of exposure; at the end of the exposure period in all vessels designated for determination of biological variables. Hardness and ammonia should be measured in the controls and one test vessel at the highest concentration at the start and the end of the test.

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DATA AND REPORTING

Treatment of results

42. The purpose of this test is to determine the effect of the test chemical on the development rate, the number of fully emerged males, the number of fully emerged females as well as the total number of fully emerged midges, or in the case of the 10-day test effects on survival and weight of the larvae. If there are no indications of statistically different sensitivities of sexes, male and female results may be pooled for statistical analyses. The sensitivity differences between sexes can be statistically judged by, for example, a χ^2-r × 2 table test. Larval survival and mean individual dry weight per vessel should be determined after 10 days where required.

43. Effect concentrations should always be reported in terms of mg test chemical/L water and in terms of mg test chemical/kg dry sediment if the chemical is detected in the sediment. Because the decision regarding the expression of endpoints should be based on the partitioning of the chemical in the various components, it is recommended to report mass balance calculations (26) (see Annex 5). This is optional depending on the requirements in the respective regulations; it is also particularly important for chemicals that are difficult to test (concentrations poorly maintained in the test system).

44. To express the endpoints as nominal concentrations, there should be evidence that the concentration of the chemical being tested has been satisfactorily maintained in the overlying water (i.e. between 80 % and 120 % of the nominal concentration throughout the test). If the concentrations are expected to deviate by more than 20 %, all test concentrations should be measured and more frequent analyses may be carried out. In this case, the endpoints should then be expressed in terms of geometric mean measured concentrations or in time-weighted arithmetic mean measured average concentrations when the samples were taken in two or more unequal time intervals (with calculations considering also the additional intermediate analytical measurements in accordance with recommendations in Annex 2 of OECD Guidance Document on Aquatic Toxicity Testing of Difficult Test Chemicals (12) and examples in Annex 6 of OECD Guideline 211: *Daphnia magna* Reproduction Test (27). In cases when the measured concentrations deviate by more than 20 % from the initial concentrations and/or measured concentrations are expected to fall below LOQ/LOD during the duration of the test (e.g. based on analytical information derived from previous water-sediment studies, non-GLP pre-tests or range finding tests), test design with renewal periods might be needed. The endpoints could then be expressed in accordance with (12). When the aim is to simulate a pesticide spray drift event, endpoints could be expressed as measured initial concentrations. Note that more frequent analytical measurements may also be carried out in this case.

45. To convert concentration data expressed e.g. as mg/kg dry sediment into mg/kg organic carbon (OC), the dry sediment concentration should be divided by the percent TOC (expressed as decimal), as follows:

$$mg/kg\ OC = \frac{mg/kg\ dry\ sediment}{kg\ OC/kg\ dry\ sediment}$$

46. To compute a point estimate for the EC10 and EC50 or any other ECx values, the per-vessel statistics may be used as true replicates. In calculating a confidence interval for any ECx 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, ECx values should be calculated after the response is

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transformed back to the original value. Further information on statistics are given in OECD Document 54 on the Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application (28).

47. When the statistical analysis aims at determining the NOEC/LOEC by hypothesis testing, the variability among vessels needs to be taken into account, for example by a nested ANOVA. Alternatively, more robust tests (29) can be appropriate in situations where there are violations of the usual ANOVA assumptions.

Emergence ratio

48. Emergence ratios are quantal data, and can be analysed by the Cochran-Armitage test applied in step-down manner where a monotonic dose-response is expected and these data are consistent with this expectation. If not, a Fisher's exact or Mantel-Haentzal test with Bonferroni-Holm adjusted p-values can be used. If there is evidence of greater variability between replicates within the same concentration than a binomial distribution would indicate (often referenced as "extra-binomial" variation), then a robust Cochran-Armitage or Fisher exact test such as proposed in (29), should be used. The CPFISH (closure principle and Fisher–Freeman–Halton (24) test may also be used for evaluation of emergence data (see paragraph 21).

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

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

where:

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

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

50. 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.

51. For emergence ratios, ECx-values are calculated using regression analysis (e.g. probit (30), logit, Weibull, appropriate commercial software etc.). If regression analysis fails (e.g. when there are less than two partial responses), other non-parametric methods such as moving average or simple interpolation are used.

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Development rate

52. The mean development time represents the mean time span between the introduction of larvae (day 0 of the test) and the emergence of the experimental cohort of midges. (For the calculation of the true development time, the age of larvae at the time of introduction should be considered). The development rate is the reciprocal of the development time (unit: 1/day) and represents that portion of larval development which takes place per day. The development rate is preferred for the evaluation of these sediment toxicity studies as its variance is lower, and it is more homogeneous and closer to normal distribution as compared to development time. Hence, powerful parametric test procedures may be used with development rate rather than with development time. For development rate as a continuous response, ECx-values can be estimated by using regression analysis (e.g. (31)(32)).

53. For the following statistical tests, the number of midges observed on inspection day x are assumed to be emerged at the mean of the time interval between day x and day x–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

$$x_i = {}^{1}\!\Big/\!\left(day_i - \frac{1_i}{2}\right)$$

where:

- day_i : inspection day (days since application)
- l_i : length of inspection interval i (days, usually 1 day)

Test report

54. The test report should at least provide the following information:

Test chemical:

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

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

Test species:

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

Test conditions:

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

Results:

- the nominal test concentrations, the measured test concentrations and the results of all analyses to determine the concentration of the test chemical in the test vessel, including assessment of mass balance if appropriate; if measurements in sediments are not performed, justification and results of the water/sediment study used to assess partitioning of the test chemical including conditions (e.g. sediment to water ratio, type of application, organic carbon content of sediment);
- water quality within the test vessels, i.e. pH, temperature, dissolved oxygen, hardness and ammonia;
- replacement of evaporated test water, if any;
- number of emerged male midges, number of emerged female midges per vessel and per day;
- number of larvae which failed to emerge as midges per vessel;
- mean individual dry weight of larvae per vessel, and per instar, if appropriate;
- percent emergence per replicate and test concentration (male and female midges pooled);

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- mean development rate of fully emerged midges per replicate and treatment rate (male and female midges pooled);
- estimates of toxic endpoints for example ECx (and associated confidence intervals), NOEC and/or LOEC, and the statistical methods used for their determination;
- discussion of the results, including any influence on the outcome of the test resulting from deviations from this Guideline.

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ANNEX 1 - DEFINITIONS

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

- 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 chemical.
- The stabilisation period is the time used after spiking to allow partitioning of the test chemical between the solid phase, the pore water and the overlying water; it takes place after preparation of the spiked sediment – water system and ideally under exposure conditions (e.g. temperature and aeration).
- Formulated sediment or reconstituted, artificial or synthetic sediment, is a mixture of materials used to mimic the physical components of a natural sediment.
- Growth: increase in dry weight of test organisms during the experiment and expressed as mean dry weight per surviving amphipod.
- Overlying water is the water placed over sediment in the test vessel.
- 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.

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ANNEX 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 lx), 8 hours dark. It has been reported that air humidity of less than 60 % RH can impede reproduction.

Dilution water

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

Feeding larvae

4. *Chironomus* larvae should be fed with a fish flake food (TetraMin®, 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 must be carefully monitored. Too little food will cause emigration of the larvae towards the water column, and too much food will cause increased microbial activity and reduced oxygen concentrations. Both conditions can result in reduced growth rates.

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

Feeding emerged adults

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

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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.

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 $NaSiO_3$ 5 H_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.

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

Stock solutions (I)	Amount (mg) made up to 1 litre of deionised water	To prepare the combined stock solution (II): mix the following amounts (ml) of stock solutions (I) and make up to 1 litre of deionised water		Final concentrations in test solutions (mg/l)	
		M4	M7	M4	M7
H3BO3 (1)	57190	1.0	0.25	2.86	0.715
$MnCl_2$ 4 H_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
$SrCl_2$ 6 H_2O (1)	3040	1.0	0.25	0.152	0.038
NaBr (1)	320	1.0	0.25	0.016	0.004
Na2MoO4 2 H2O (1)	1260	1.0	0.25	0.063	0.016
$CuCl_2$ 2 H_2O (1)	335	1.0	0.25	0.017	0.004
$ZnCl_2$	260	1.0	1.0	0.013	0.013
$CoCl_2$ 6 H_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
Na2EDTA 2 H2O (1)(2)	5000	20.0	5.0	2.5	0.625
FeSO4 7 H2O (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)
$CaCl_2$ 2 H_2O	293800	1.0	293.8
$MgSO_4$ 7 H_2O	246600	0.5	123.3
KCl	58000	0.1	5.8
$NaHCO_3$	64800	1.0	64.8
$NaSiO_3$ 9 H_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

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Table 3: Vitamin stock solutions 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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ANNEX 3 - PREPARATION OF FORMULATED SEDIMENT

Sediment composition

The composition of the formulated sediment should be as follows:

Constituent	Characteristics	% of sediment dry weight
Peat	e.g. 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	CaCO3, pulverised, chemically pure	0.05-1
Water	Conductivity 10 µS/cm	30-50

Preparation

1. 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 a 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

2. 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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ANNEX 4 - CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

SUBSTANCE	CONCENTRATIONS
Particulate matter	< 20 mg/l
Total organic carbon	< 2 mg/l
Unionised ammonia	< 1 µg/l
Hardness as $CaCO_3$	< 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 must not be used in this situation).

**Please note that this item is only to be checked in case natural or tap water is used.

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ANNEX 5 - MASS BALANCE CALCULATIONS FOR SEDIMENT-WATER TOXICITY TESTS; EXAMPLE FOR SPIKED WATER TEST PERFORMED WITH *CHIRONOMUS* SP.

1. The starting point for a mass balance calculation for a study with sediment-dweller organisms in sediment-water tests using spiked sediment (e.g. OECD TG 218) or spiked water (e.g. OECD TG 219) is the measured concentrations of the test chemical in the test system considering the concentrations at the beginning and end of the study as well any additional intermediate concentrations available.

2. The mass balance calculation in the case of studies with sediment-dwellers with spiked water (OECD TG 219) is illustrated below by an example for a chemical unstable in water (e.g. a pesticide).

3. The information on the analytical determination of the chemical needed for the mass balance calculation is given for the example in Table 1. The measurements in this case took place at three time points (1 hour, 7 days and 28 days). For simplicity, they are reported here only at the lowest and highest concentrations tested. However, measurements at additional tested concentrations are recommended, particularly in the case of chemicals that are difficult to test (e.g. when concentrations are poorly maintained in the test system).

4. For the example below, the test system contains an amount of sediment of 0.1 kg and a volume of overlying water of 400 ml; it is assumed that the pore water has a volume of 10 ml. Based on this information, it is possible to calculate the amount of chemical that partitions to the various compartments by multiplying the concentration by the volume/amount of water/sediment and to calculate the percentage in each compartment with respect to that initially contained in the spiked sediment.

5. In order to assess the fate of the test chemical in the sediment/water system, the mass balance calculation was performed for each time point for which measurements were available (see Table 2B).

Example of a test chemical unstable in water

Table 1: Measured concentrations of the test chemical in different compartments of a spiked water study with *Chironomus riparius* performed in line with OECD TG 219.

Measured concentrations-(mg a.s/kg dry sediment or µg a.s./L)									
	day 0 (start of exposure)			**7 days**			**28 days**		
Nominal (µg a.s./l)	Overlying water (µg a.s./l)	Pore water (µg a.s./l)	Sediment (µg a.s./kg)	Overlying water (µg a.s./l)	Pore water (µg a.s./l)	Sediment (µg a.s./kg)	Overlying water (µg a.s./l)	Pore water (µg a.s./l)	Sediment (µg a.s./kg)
Control	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
1.5	0.91	0.08	1.5	0.23	0.02	1.3	0.04	0.01	1.3
10	6.6	0.2	8.2	1.9	0.1	8.1	0.45	0.01	7.3

a.s.: active substance (test chemical); LOQ: limit of quantification

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Table 2: Example of mass balance calculations for the test chemical. The amount in both compartments – sediment and water (overlying water)- is expressed in terms of total amounts (Table 2A) and in percentage (Table 2B) of test chemical with respect to the amount initially contained in the system (measured in water and sediment).

Table 2A

Amounts (µg) calculated from measured concentrations									
	day 0 (start of exposure)			7 days			28 days		
Nominal (µg)	Overlying water (µg)*	Pore water (µg)*	Sediment (µg)*	Overlying water (µg)	Pore water (µg)	Sediment (µg)	Overlying water (µg)	Pore water (µg)	Sediment (µg)
Control	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
1.5	0.364	0.0008	0.15	0.092	0.0002	0.13	0.016	0.0001	0.13
10	2.64	0.002	0.82	0.76	0.001	0.81	0.18	0.0001	0.73

Calculated considering *100 g sediment, 400 ml overlying water, 10 ml pore water

Table 2B

Mass balance									
	day 0 (start of exposure)			7 days			28 days		
Nominal (µg)	Overlying water (%)	Pore water (%)	Sediment (%)	Overlying water (%)	Pore water (%)	Sediment (%)	Overlying water (%)	Pore water (%)	Sediment (%)
Control	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
1.5	70.707	0.156	29.138	17.871	0.039	25.253	3.108	0.019	25.253
10	76.256	0.058	23.686	21.953	0.029	23.397	5.199	0.003	21.086

6. In the example, the test chemical partition to the sediment is up to 29.1% in the first hour, while at the end of the study (i.e. at 28 days) the chemical is still present in the sediment at levels of up to 25.2% of that initially measured.

7. In the case of the water compartment, the chemical is measured mostly in the overlying water in the first hour (i.e. at levels up to 76.2%). At the end of the study, the chemical is only present in the water at levels up to 5.2% of that initially measured.

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8. During this test, both dissipation to the sediment and degradation occurred; the total amount of the test chemical in the test system in the study accounts for up to ca. 27 % of the total amount initially measured.

9. In such a case, it is needed to express the endpoints in terms of mean measured concentrations (preferably time-weighted arithmetic mean measured concentrations rather than geometric mean measured concentrations, as the time intervals are unequal) as the concentrations in water were not maintained within 20 % of the initial measured concentrations. It is also highly recommended to analyse all tested concentrations and any further additional intermediate measurements. It is additionally recommended that the key endpoints are presented in terms of mg of test chemical per l of water and in mg of test chemical per kg of dry sediment. This would ensure that both exposure via water and sediment are covered for sediment-dwellers.

10. See also Appendix G in EFSA, 2019.

Reference

- EFSA (2019). Pesticide Peer Review meeting on general recurring issues in ecotoxicology (EFSA Supporting publication 2019:EN-1673)

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