Revised draft new Test Guideline for the Hyalella azteca Bioconcentration Test (HYBIT)

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Draft Test Guideline

Hyalella azteca Bioconcentration Test (HYBIT)

INTRODUCTION

1. This Guideline provides a non-vertebrate test to derive the bioconcentration potential of chemicals. *In vivo* fish bioconcentration factors (BCFs) determined in accordance with the OECD test Guideline 305 (1) are used in many regulations for bioaccumulation assessment. However, not all regulatory frameworks permit vertebrate testing. Also, animal welfare reasons, potential cost reduction and further technical options for testing difficult to test chemicals are aspects that may favour the application of alternative test methods.

2. This Guideline describes a procedure for characterising the bioaccumulation potential of chemicals in the benthic freshwater amphipod *H. azteca*. It has been developed in such a way that it is as close as possible to the concept described in the OECD TG 305 (I), with the exception of a minimized exposure design.

3. In addition to the established flow-through regime commonly applied in bioconcentration studies, semi-static regimes are permissible as options in studies carried out according to this Guideline. Both regimes have been validated as part of an international ring trial (OECD validation report, in prep).

4. The aqueous exposure test is most applicable to stable organic chemicals with log K_{ow} values between 1.5 and 6.0 (3), but may still be used with strongly hydrophobic chemicals (having log $K_{ow} > 6.0$), if a stable and fully dissolved concentration of the test chemical in water can be demonstrated (18). The log K_{ow} measurement is based on the steady-state thermodynamics of solutes, and so the log K_{ow} test may not be suitable to characterise bioaccumulation of chemicals that exist as suspensions.

5. The bioconcentration test is generally based on measuring the bioaccumulation of parent compound. Radiolabelled test chemicals can enable a sensitive analysis of water and tissue samples. Separation procedures e.g. TLC or HPLC should be employed prior to radio-detection to enable quantitative analysis of the parent and transformation products. Peaks associated with the parent and transformation products should be verified using non-labelled certified reference standards. When separation techniques are applied, a BCF determination for the parent test chemical should be based upon the concentration of the parent test chemical in *H. azteca* and not upon total radioactive residues. If, total radioactive residues (TRR) are determined (e.g. by combustion or tissue solubilisation), the radioactivity and therefore the BCF is based on the total radioactivity associated with parent test chemical, retained metabolites and assimilated carbon. BCF values based on TRR maybe overly conservative and not, directly comparable to a BCF derived where an extraction method has been employed followed by specific chemical analysis of the parent test chemical only.

6. The decision on whether to conduct a flow-through or semi-static exposure experiment should be based on the opportunity to maintain stable exposure concentrations in the water phase during uptake (*cf.* paragraph 17). Important factors that may influence application choice should be considered, including the test chemical's potential for adsorption to test vessels and apparatus, its stability in aqueous solution, etc. Information on such practical aspects may be available from other aquatic toxicity tests. If no information is available, a pre-test should be conducted to confirm the suitability of the selected exposure regime (18).

7. It should be verified that the aqueous exposure concentration(s) to be applied are within the aqueous solubility range of the test chemical in the test media. Different methods for maintaining stable concentrations of the dissolved test chemical can be used, such as the use of stock solutions or passive dosing systems (*e.g.* column elution method) (18). It should be demonstrated that stable concentrations can be maintained and the test media are not altered from the media recommended in Annex 3 and fulfil the requirements listed in paragraph 20.

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PRINCIPLE OF THE TEST

8. The test consists of two phases: the exposure (uptake) and post-exposure (depuration) phases. During the uptake phase, a group of *H. azteca* is exposed to the test chemical at one or more chosen concentrations, depending on the properties of the test chemical (*cf.* paragraphs 14 and 38). They are then transferred to a medium free of the test chemical for the depuration phase. The concentration of the test chemical in/on the analysed *H. azteca* is followed through both phases of the test. Parameters which characterise the bioaccumulation potential include the uptake rate constant (k_1), the depuration rate constant (k_2), the steady-state bioconcentration factor (BCF_{SS}) and the kinetic bioconcentration factor (BCF_K). In addition to the exposed group, a procedural water control should be included which is held under identical conditions (including sampling), to relate possible adverse effects observed in the bioconcentration test to a matching control group. If the use of solvent is required (*cf*. paragraph 23), one control containing the solvent, instead of the procedural water control, should be run in addition to the treatment group(s).

9. In the bioconcentration test, the uptake phase is usually run for 2 – 14 days (*cf.* paragraph 10 and paragraph 28). A prediction of the length of the uptake phase and the time to steady-state can be made from empirical knowledge (2) and pre-test results (*cf.* paragraph 27). The depuration period starts when the *H. azteca* are no longer exposed to the test chemical, by transferring the *H. azteca* to test medium without test chemical in a clean vessel. Where possible the bioconcentration factor is calculated preferably as a kinetic bioconcentration factor (BCF_K), which is estimated as the ratio of the rate constants of uptake (k_1) and depuration (k_2) assuming first order kinetics (c*f*. Annex 1 definitions and units).

10. The bioconcentration factor should also be calculated as the ratio of concentration in the *H. azteca* (C_H) and in the water (C_w) at apparent steady-state (BCF_{ss}; *cf.* Annex 5). In principle, the BCF_{ss} should be comparable to the BCF_k but deviations may occur if steady-state was uncertain. If a 'steady-state' is not achieved within 14 days the calculation of a BCF is based exclusively on the kinetic approach (*cf.* Annex 5).

11. The uptake rate constant, the depuration (loss) rate constant (or constants, where more complex models are involved), the bioconcentration factor (steady-state and/or kinetic), and, where possible, the confidence limits of each of these parameters are calculated from the model that best describes the measured concentrations of the test chemical in *H. azteca* and water (*cf.* Annex 5).

12. An increase in the mass of the *H. azteca* during the test can be neglected since adult individuals are used in the test (OECD validation report in prep). A correction of the kinetic BCF for the so-called growth dilution is therefore not necessary.

13. The BCF is based on the total concentration of the parent substance in *H. azteca* (*i.e.* per total wet weight of the sampled *H. azteca*). Since, for many organic chemicals, there is a clear relationship between the potential for bioconcentration and hydrophobicity, there is also a corresponding relationship between the lipid content of the test *H. azteca* and the observed bioconcentration of such chemicals. Thus, to reduce this source of variability in test results for those test chemicals with high lipophilicity (*i.e.* with log $K_{ow} > 3$), bioconcentration should be expressed as normalised to *H. azteca* with a default 3% lipid content (based on whole body wet weight). The lipid content of lab-raised *H. azteca* is usually in the range of 1-3% (w/w) but may be higher in field caught *H. azteca* (1, 4). Lipid measurements shall be carried out for amphipods collected directly from the study (*cf*. Annex 6). This is necessary to provide a basis from which results for different chemicals and studies can be compared against one another.

INFORMATION ON THE TEST CHEMICAL

14. Before carrying out the bioaccumulation test, the following information about the test chemical should be known:

(a) Sensitivity of the analytical technique for measuring tissue and aqueous concentrations of the test chemical.

(b) Solubility in water [OECD TG 105; (6)]; this has to be determined in accordance with a method that is appropriate for the (estimated) range of the solubility to obtain a reliable value. For hydrophobic test chemicals, this will generally be the column elution method.

(c) *n*-Octanol-water partition coefficient, K_{ow} (or where relevant LogP) [OECD TGs 107 (7), 117 (8), 123 (9)]; or other suitable information on partitioning behaviour (*e.g.* sorption to organic carbon, K_{oc});

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this has to be determined in accordance with a method that is appropriate for the (estimated) range of the K_{ow} to obtain a reliable value. For hydrophobic test chemicals, this will generally be the slow-stirring method [OECD TG 123 (9)];

(d) Test chemical stability in water (hydrolysis [OECD TG 111 (10)]);

(e) Information on phototransformation relevant for the light conditions in the test (11);

(f) Surface tension (*i.e.* for chemicals where the log K_{ow} cannot be determined) [OECD TG 115 (12)];

(g) Vapour pressure [OECD TG 104 (13)];

(h) Any information on biotic or abiotic degradation in water, including ready biodegradability [OECD TGs 301 A to F (14), 310 (15)], where appropriate;

(i) Information on metabolites: structure, log K_{ow}, formation and degradability, where appropriate;

(j) Acid dissociation constant (pK_a) for test chemicals that might ionise. If possible the pH of the test water should be adjusted to ensure that the test chemical is in the unionised form in the test if compatible with *H. azteca*.

15. An exposure concentration should be selected that does not cause adverse effects in the test species. If this information is not available, a toxicity range finder is conducted as preliminary experiment (*cf.* paragraph 39; Annex 9). Alternatively, other toxicity endpoints estimated from invertebrate tests can be used (e.g. OECD TG 202 (16), OECD TG 211 (17)).

16. An appropriate analytical method of known accuracy, precision and sensitivity should be available for the quantification of the test chemical in the test solutions and in biological material, together with details of sample preparation and storage. The analytical quantification limit of the test chemical in both water and *H. azteca* tissues should also be known. The test chemical used should be of the highest purity (*e.g.* preferably > 98%). When a radiolabelled test chemical is used the percentage of radioactivity associated with impurities should be known.

VALIDITY OF THE TEST

17. For a test to be valid the following criteria should be met:

- The water temperature variation in the test vessels should be within the range 23±2°C. However, the temperature should not vary by more than ±1°C within 24 hours;
- The concentration of dissolved oxygen does not fall below 50% saturation at any sampling time;
- The concentration of the test chemical in the test vessels is maintained within ± 20% of the mean of the measured values during the uptake phase;
- The concentration of the test chemical is below its limit of solubility in the test medium (taking into account the effect that the test media composition may have on effective solubility);
- Mortality of *H. azteca* is less than 20% at the end of the test in both the control and treatment group(s), that means all samples could be collected in the course of the study and there are still animals left in each test vessel (*cf.* Annex 4).

If a (minor) deviation from the test validity criteria is observed, the consequences should be considered in relation to the reliability of the test results and these deviations and considerations should be included in the test report.

REFERENCE CHEMICALS

18. The use of reference chemicals of known bioconcentration potential (and low metabolism) would be useful in checking the experimental procedure, when required (*e.g.* when a laboratory has no previous experience with the test or experimental conditions have been changed). For a flow-through approach with compounds with elevated hydrophobicity (Log K_{ow} 5 - 6), hexachlorobenzene (HCB) would be an appropriate reference chemical. For a semi-static approach with moderately lipophilic compounds, chemicals like prochloraz would be suitable. Both chemicals were applied during the HYBIT ring trial, the results of which provide information on the BCF ranges to be expected (OECD validation report, in prep.). However, other chemicals can also be used with an appropriate reasoning.

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DESCRIPTION OF THE METHOD

Apparatus

19. Care should be taken to avoid the use of materials, for all parts of the equipment, that can dissolve, sorb or leach and have an adverse effect on *H. azteca*. Standard rectangular glass aquariums with a volume capacity of 20 L can be used as test vessels. In order to provide a refuge for *H. azteca*, a mesh of stainless steel (850-1000 µm, tunnel shaped) can be added to the aquarium during the test. The use of soft plastic tubing should be minimised. Use Teflon®, stainless steel and/or glass tubing where it is possible. It is preferable to expose test systems to concentrations of the test chemical to be used in the study for at least three days to demonstrate by daily analytical measurements the maintenance of stable exposure concentrations prior to the introduction of test organisms.

Water

20. Any laboratory test water acceptable under OECD criteria (*cf.* Annex 3) can be used as dilution water in a test. The dilution water, which is the water that is mixed with the test chemical before entering the test vessel (*cf.* paragraph 21-22), should be of a quality that will allow the survival of *H. azteca* for the duration of the acclimation and test periods without them showing any abnormal appearance or behaviour. It should be demonstrated that the *H. azteca* can survive, grow and reproduce in the dilution water (natural water, tap water or reconstituted medium), e.g. in laboratory culture or a life-cycle toxicity test. An acclimation phase might be required to exclude mortality especially if a media change is performed between *H. azteca* husbandry and test performance. The dilution water should be characterised at least by pH, hardness, total solids, total organic carbon (TOC) and also ammonium, nitrite and alkalinity. Some chemical and physical characteristics of an acceptable dilution water are given in Annex 3.

21. The dilution water should be of constant quality during the period of a test. The pH value should be within the range 6.0 to 8.5 at test start, but during a given test it should be within a range of ± 0.5 pH units. If natural water or tap water is used instead of a reconstituted medium, samples should be taken at regular intervals for determination of selected water parameters as given in Annex 3, in order to ensure that the dilution water will not unduly influence the test result (for example, by complexation of the test chemical) or adversely affect the performance of the stock of *H. azteca*. Measurements of the charactersitics of the dilution water should be performed at least twice a year or whenever it is suspected that water parameters may have changed significantly. If using natural water, special care should be taken considering the characteristics mentioned.

22. The natural particle content as well as the TOC of the dilution water should be as low as possible to avoid adsorption of the test chemical to organic matter which may reduce its bioavailability and thus result in an underestimation of the BCF. The maximum acceptable value is 5 mg/L for particulate matter (dry matter, not passing a 0.45 µm filter) and 2 mg/L for TOC (*cf.* Annex 3). If necessary, the dilution water should be filtered before use.

Test Solutions

23. A stock solution of the test chemical should be prepared at a suitable concentration. The stock solution should preferably be prepared by simply mixing or agitating the test chemical in the dilution water. An alternative that may be appropriate in some cases is the use of a solid phase desorption dosing system (22). The use of solvents (solubilising agents) is generally not recommended (*cf.* (18)). However, the use of these materials may still be required in order to produce a suitably concentrated stock solution, but every effort should be made to minimise the use of such materials. Solvents which may be used are acetone, ethanol, methanol, dimethyl formamide and triethylene glycol. The solvent concentration in the final test medium should be the same in all treatments (i.e. regardless of test chemical concentration) and should not exceed the corresponding toxicity thresholds determined for the solvent under the test conditions. The maximum level is a concentration of 100 mg/L (or 0.1 mL/L). It is unlikely that a solvent concentration of 100 mg/L will significantly alter the maximum dissolved concentration of the test chemical which can be achieved in the medium (18). The test chemical concentration should be below the solubility limit of the test chemical in the test media in spite of the use of a solvent or solubilising agent. The solvent's contribution (together with the test chemical) to the overall content of organic carbon in the test water should be known. Throughout the test, the concentration of total organic carbon in the test vessels should not exceed the concentration of organic carbon originating from the test chemical, and solvent or solubilising agent (1), if used, by more than 10 mg/L (± 20%). Organic matter content can have a significant effect on the amount of freely dissolved test chemical, especially for highly lipophilic chemicals (19). Solid-phase microextraction (SPME) (20, 21) can provide important information on the ratio between bound and freely dissolved compounds, of which the latter is assumed to represent the bioavailable fraction. Care should be taken when using readily biodegradable solvents (e.g. methanol) as these can cause problems with biofilm formation

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which can lead to dietary transfer of the test chemical and thus alter the uptake kinetics. If biofilm is formed it must be removed consistently or, if this is not possible, flow-through tests with a solvent-free dosing system such as solid phase desorption dosing (22) or passive dosing (23) should be considered (*cf.* paragraph 24).

24. For flow-through tests, a system which continuously dispenses and dilutes a stock solution of the test chemical (*e.g.* metering pump, proportional diluter, saturator system) or a solid phase desorption dosing system is required to deliver the test concentrations to the test vessels. Preferably allow at least five-volume replacements through each test vessel per day. The flow-through mode is to be preferred, but a semi-static technique may be used provided that the validity criteria are satisfied (*cf.* paragraph 17).
The flow rates of stock solutions and dilution water should be checked both 48 hours before and then at least daily during the test. It is recommended to check the flow rate in each test vessel (which should not vary by more than 20% between consecutive measurements) to ensure constant exposure conditions.

Holding of *H. azteca*

25. The *H. azteca* used for the bioconcentration experiments should ideally be derived from an in-house laboratory culture. If in-house husbandry is not possible then amphipods can be purchased from commercial sources. In this instance the amphipods need to be acclimatized to the laboratory conditions (media, feed and temperature) for at least one month. The recommended procedure for the laboratory husbandry of *H. azteca* is based on an established protocol using a culture medium containing essential mineral nutrients (24). The feed recommended for laboratory husbandry or acclimatization to laboratory conditions, differs from that used during the test. An example of suitable husbandry and culture conditions is given in Annex 2.

26. *H. azteca* used in tests should be free from observable diseases and abnormalities. Any diseased amphipods should be discarded. *H. azteca* should not receive treatment for disease in the two weeks preceding the test, or during the test.

PERFORMANCE OF THE TEST

Preliminary test

27. It may be useful to conduct a preliminary experiment in order to optimise the test conditions of the definitive test, *e.g.* exposure regime, selection of test chemical concentration(s), duration of the uptake and depuration phases. A proposal for a preliminary toxicity test is given in Annex 9.

Conditions of Exposure

Duration of uptake phase

28. In contrast to fish bioconcentration studies, a prediction of the duration of the uptake phase cannot be made based on equations (1), but can be estimated based on log K_{OW} values as previously described (2):

- Low to moderate hydrophobicity (Log K_{OW} < 4): 3 – 4 days
- Moderate to elevated hydrophobicity (Log K_{OW} 4 – 5): 4 – 10 days
- High hydrophobicity (Log K_{OW} > 5): more than 10 days

29. The uptake phase should be run for a time period sufficiently long to guarantee that steady-state has been reached. A steady-state is reached in the plot of test chemical in *H. azteca* (C_H) against time when the curve reaches a plateau and three successive analyses of C_H made on samples taken at sufficiently large intervals relative to the uptake phase are within ± 20% of each other, and there is no significant increase of C_H between the first and last successive analysis. For test chemicals with high hydrophobicity requiring an uptake phase of 10 days or more to reach steady-state, a sampling interval of at least 48 hours between each of the last three sampling points should be established to allow evaluation of steady-state conditions. For substances with low to moderate hydrophobicity a shorter sampling interval (e.g. 12 to 24 hours between the last three sampling points) should be appropriate to evaluate steady-state conditions (cf. Annex 4). If steady-state has not been reached with 14 days of exposure, the BCF is calculated using the kinetic approach, which does not depend on steady-state. However, the test chemical concentration in *H. azteca* at the end of the uptake phase needs to be sufficiently high to ensure a reliable estimation of the uptake and elimination rate constants. In few cases no measurable uptake of the test substance may have occurred at the end of the uptake period. If it can be demonstrated that: i) the validity criteria in paragraph 17 are fulfilled; and ii) lack of uptake is not due to some other shortcoming of the test (e.g. uptake duration not long enough, lack of sensitivity of the analytical method, etc.); it may be possible to terminate the study without the need to re-run it.

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Duration of the depuration phase

30. The duration of the depuration phase should be at least as long as the uptake phase and should be long enough to allow an appropriate reduction (e.g. 95%) in the body burden of the test chemical (mean steady-state tissue concentration) to occur. If the time required to reach 95% loss is impractically long, the study can be terminated after twice the duration of the uptake phase provided that the concentration of test chemical in the amphipods is at least lower than 10% (>90% loss) of the previously measured steady-state tissue concentration. If preliminary knowledge about the depuration behaviour of the test chemical is available, the depuration phase can be shortened or extended appropriately. If a substance is depurated very slowly such that an exact half-life may not be determined in the depuration phase, the information may still be sufficient for assessment purposes to indicate a high level of bioaccumulation.

Numbers of H. azteca in the test

31. The numbers of *H. azteca* per test (concentration) should be selected in a way that three replicate samples with a minimum of 20 amphipods each are available at each sampling time (i.e. a total minimum number of 60 amphipods per sampling time). The minimum of 20 individuals per sample is needed to ensure a sufficient biomass for the chemical analysis. An example sampling scheme is provided in Annex 4. A higher amount of pooled *H. azteca* per replicate sample will be necessary if required by the analytical procedure. An increased number of samples per sampling time might be required if additional analyses are intended. The lipid content should be determined on additional *H. azteca* that have been sampled from the same test vessel concurrent with the amphipods used to determine the concentration of the test chemical. An appropriate number of amphipods needs to be sampled (e.g. >50 mg/pooled sample) to ensure an accurate determination of the lipid content. (*cf.* Annex 6).

32. Only sexually mature males (about 8 weeks old) are used to avoid reproduction during the test. Male *H. azteca* are used due to their more uniform size and lipid content compared to female *H. azteca*. The sexing procedure to select sexually mature male *H. azteca* is described in Annex 7. Test amphipods should not be older than 6 months at the start of a study.

33. *H. azteca* used within one study should derive from the same source and culture batch. The approximate age of the test amphipods should be recorded.

Loading

34. The test vessel should be filled with a sufficient volume (e.g. 15 L) of test medium to provide a high water-to-biomass ratio (e.g. 100 animals / L) in the test system. In this way, a potential reduction in the concentration of the test compound in water caused by the addition of the *H. azteca* at the start of the test can be neglected even if the water-to-biomass ratio is decreased to a lower value (e.g. 150 animals/L). The same applies to the decrease in dissolved oxygen concentration often observed in bioconcentration studies with fish.

Feeding

35. During the test period, *H. azteca* are fed with ground agar-agar-bound fish flakes (so called Decotabs). The feed is applied daily. At the beginning of the study, a higher amount of feed is supplied. Five cubes suffice for approx. 1000 *H. azteca*. With the number of test amphipods being reduced in the course of the study, the number of applied Decotab cubes is reduced accordingly. Uneaten Decotabs should be removed before adding fresh feed. Before the application, each cube is subdivided in smaller portions. Using Decotabs as feed will ensure that the *H. azteca* receive an appropriate diet of known lipid and total protein content in an amount sufficient to keep them in a healthy condition and to maintain body weight. A detailed protocol for preparing the Decotabs is described in Annex 8. Decotab-feeding has been shown to be the most appropriate feeding approach for *H. azteca* in BCF studies (5) and is thus the preferred feeding method for the HYBIT tests, however, this does not prevent other suitable methods from being used, provided they have demonstrated to fulfil the purpose equivalently, e.g. by compliance with the respective validity criteria.

36. The test vessels need to be kept as clean as possible throughout the test to keep the concentration of organic matter (e.g. amphipod excreta and feed residues) as low as possible. The test vessel cleaning routine will depend on the chosen test setup and exposure method. Decotab-feeding has been shown to have no significant effect on water quality. No increase in TOC in the test vessels above the recommended threshold values (e.g. 10 mg/L) was observed (OECD validation report, in prep.).

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Light and temperature

37. A 16-hour photoperiod and a temperature range of 23°C (± 2°C) are recommended. Wide-spectrum fluorescent lamps (840 K) should be used with LED light sources representing a potential alternative to fluorescent lighting. Light intensity is measured at the water surface of the vessel and should not exceed 8-16 $\mu E \bullet m^{-2} \bullet s^{-1}$ or 500 to 1000 lux.

Test concentrations

38. Similar to the OECD test guideline 305, the test was originally designed for neutral organic chemicals. For this type of chemicals, the exposure to a single concentration is expected to be sufficient, as no concentration effects are expected on bioconcentration provided that concentrations are chosen well below solubility and toxicity limits. If test chemicals outside this domain are tested, or other indications of possible concentration dependence are known, the test has to be run with two or more concentrations. If only one concentration is tested, justification for the use of one concentration should be given. The tested concentration should be as low as is practical or technically possible (*i.e.* not close to the solubility limit).

39. The selected test chemical concentration for *H. azteca* should be below its chronic effect level or 1% of its acute asymptotic LC_{50}. A proposal for a toxicity test as preliminary experiment for HYBIT tests is described in Annex 9. Alternatively, a toxicity range finder can be run over the expected time of the uptake period to estimate the NOEC level for the following HYBIT test. Generally, the exposure concentration should be at least an order of magnitude above its limit of quantification in water as determined by the analytical method. In addition, care should be taken that the test concentration is below the solubility limit of the test chemical in the test media. If it is technically not possible to reduce the concentrations to such low levels, the use of two concentrations is always recommended.

Control

40. A procedural water control should be included to demonstrate that the test conditions provided are appropriate for *H. azteca*, ensuring a sufficient survival throughout the study. *H. azteca* of the control group should be sampled in accordance to the test group to keep the stocking densities in both groups on the same level. If a solubilising agent has been used to prepare an appropriately concentrated stock solution, the control group should be treated in exactly the same way, but without the test chemical, so that the solvent concentration is the same in the procedural water control (solvent control) and all treatment group(s). If test conditions require pH adjustment of the dilution water the pH of the procedural water control should be adjusted accordingly.

Frequency of Water Quality Measurements

41. During the test, dissolved oxygen, total organic carbon (TOC), total hardness, pH and temperature are measured in all test vessels. Dissolved oxygen, pH, and temperature are measured and recorded daily. TOC is measured at the beginning of the test before addition of the *H. azteca*, at the end of the uptake phase and at the beginning and end of the depuration phase. If an organic solvent is used in the uptake phase, an increased number of measuring intervals for TOC may be applied. Hardness should be determined once during each test.

Sampling and Analysis of *H. azteca* and Water

H. azteca and water sampling schedule

42. Water samples are collected from the test vessel for the determination of test chemical concentration before addition of the *H. azteca* to the test system and during both, uptake and depuration phases. The duplicated water samples are taken before feeding, at the same time as the *H. azteca* sampling. During the uptake phase, the concentrations of test chemical should be determined in order to check compliance with the validity criteria (*cf.* paragraph 17). The concentration of the test chemical measured in the test solution should be maintained within ± 20% of the mean of the concentrations measured during the uptake phase. During the depuration phase, additional water samples should be taken as a precautionary measure to confirm the absence of contamination in the test system during the depuration phase. Under semi-static conditions water samples should be taken at least prior to the first medium exchange following the start of the depuration phase. In the flow-through test setup, taking a single water sample at the start of the depuration phase (within 60 minutes following transfer of all remaining *H. azteca* to water free of test chemical) is sufficient, provided that the results of the water analysis show that the test chemical is not detected (<LOQ). If the test chemical is still detected during the depuration phase, the water exchange rate should be increased and further measurements should be performed on a daily basis until the concentration is below the limit of quantification (LOQ).

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43. In the semi-static test setup, analysis of test chemical concentration should be carried out on fresh and aged media collected throughout the uptake phase. Aged media samples are taken prior to the daily media exchange. Media exchange intervals can be shortened or extended to provide stable exposure conditions. The media exchange is carried out by preparing a (clean) vessel filled with fresh test medium (uptake phase) or dilution water (depuration phase) and transferring all remaining animals into the new vessel using a small dip net.

44. *H. azteca* are sampled on at least five occasions during the uptake phase and on at least four occasions during the depuration phase to successfully capture both, the steady-state concentrations and the kinetics. Since on some occasions it is difficult to calculate a reasonably precise estimate of the BCF value based on this number of samples (especially when other than simple first order uptake and depuration kinetics are indicated), it may be advisable to take samples at a higher frequency in both periods (*cf.* Annex 4). The sampling time points will depend on the test chemical characteristic as well as on the chosen test setup. At each sampling, three independent replicates (n=3; 20 *H. azteca* pooled each) are taken from the test vessel and their wet weight determined (*cf.* paragraph 54).

45. The lipid content should be determined in amphipods collected from the test system at least at the start and end of the uptake phase and at the end of the depuration phase. At each sampling, three independent replicates (n = 3; 10 *H. azteca* pooled each) are taken from the test vessel and their wet weight determined (*cf.* paragraph 54). The number of *H. azteca* per test vessel at the start of the experiment should be adjusted accordingly. Dead or diseased *H. azteca* should not be analysed for test chemical or lipid concentration.

46. Before beginning the depuration phase, the amphipods are transferred to clean vessels. They are collected e.g. with a fine-mesh dip net, carefully rinsed with control medium and then added to the respective depuration vessel. The rinsing step is required to minimise any overestimation due to chemical that is not internalised but only adhering on the surface of the organism.

47. At the end of the experiment, the surviving amphipods are counted. For the calculation of the mortality rate, missing organisms are considered to be dead.

Sampling and sample preparation

48. Water samples for analysis are obtained e.g. by siphoning through inert tubing from a central point in the test vessel. Alternatively, water samples may be taken using a suitable pipet (e.g. 10 mL) after mixing the water in the test vessel by carefully stirring. Especially for highly hydrophobic chemicals (i.e. those chemicals with a log K_{OW} > 6) where adsorption to filter matrix or centrifugation vials could occur, collected samples should neither be subjected to filtration nor centrifugation. Instead, measures should be taken to keep the tanks as clean as possible (*c.f.* paragraph 36) and the content of total organic carbon should be monitored during both the uptake and depuration phases (*cf.* paragraph 20 and 41). To avoid possible issues with reduced bioavailability, sampling by solid phase microextraction techniques may be used for poorly soluble and highly hydrophobic substances.

49. Amphipods are collected with a fine-mesh dip net, rinsed with water and blotted dry with a lint-free tissue prior to weighing of the pooled samples.

50. All samples should be analysed preferably immediately after sampling in order to prevent degradation or other losses of the test chemical and to be able to monitor the test concentration throughout the test period. Failing immediate analysis, the samples should be stored under appropriate conditions. Before the beginning of the study, information on the proper method of storage for the particular test chemical has to be gathered – for example, deep-freezing, holding at 4°C, duration of storage, extraction, etc.

Quality of analytical method

51. The quality of the analytical results is governed essentially by the accuracy, precision and sensitivity of the analytical method used for the test chemical. Accordingly, it should be checked experimentally if the accuracy, precision and reproducibility of the analysis, as well as recovery of the test chemical from both water and amphipods are satisfactory for the particular method. Also, it should be asserted that the test chemical is not detectable in the dilution water used. If necessary, the values of test chemical concentration in water and amphipods obtained from the test for the recovery and background value of the control are corrected. Both, amphipod and water samples should be handled in such a manner as to minimise contamination and loss (e.g. resulting from adsorption by the sampling device) throughout the study.

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Analysis of H. azteca tissue samples

52. The concentration of the test chemical should be determined for each weighed pooled sample. If radiolabelled test chemicals are used in the test, it is possible to analyse for TRR (i.e. parent test chemical and metabolites). The TRR may be further separated so that the parent test chemical can be analysed separately.

53. BCF values for lipid accumulating test chemicals should be expressed as normalised to a tissue with a 3% lipid content (based on wet weight) in addition to that derived directly from the study. A suitable method should be used for determination of the amphipods' lipid content. The Smedes-method with a down-scaled protocol for small sample masses is recommended for *H. azteca* (2, 25, 26), however, this does not prevent other suitable methods from being used, provided they have demonstrated to fulfil the purpose equivalently, e.g. by compliance with the respective validity criteria. Generally, care should be taken to ensure that a suitable microbalance is used to determine the small sample masses that occur when determining the lipid content of the collected amphipods.

H. azteca weight measurement

54. The average weight of *H. azteca* (determined for each pooled replicate sample) collected at each sampling event should be measured (after careful blotting) before the chemical (or lipid) analysis is conducted. The use of an appropriate microbalance is required (*cf.* paragraph 53).

DATA AND REPORTING

Treatment of results

55. The uptake curve of the test chemical is obtained by plotting its concentration in/on *H. azteca* (C_H) in the uptake phase against time on arithmetic scales. If the curve has reached a plateau, that is, become approximately asymptotic to the time axis (*cf.* paragraph 29), calculate the steady-state BCF (BCF_{SS}) from:

$$BCF_{SS} = \frac{C_H \text{ at steady state (mean)}}{C_W \text{ as time weighted average (TWA)}} \quad \text{[Equation 1]}$$

As the mean exposure concentration (C_W) is influenced by variation over time, the time-weighted average (TWA) water concentration is more relevant and precise for bioaccumulation studies (especially under semi-static conditions), even if variation is within the appropriate validity range. The TWA C_W is calculated according to Annex 5.

56. The kinetic bioconcentration factor (BCF_K) is determined as the ratio k_1/k_2, the two first order kinetic rate constants. Rate constants k_1 and k_2 and BCF_K can be derived by simultaneously fitting both the uptake and the depuration phase (*c.f.* Annex 5, Equation A 5.6). Alternatively, k_1 and k_2 can be determined sequentially (*cf.* Annex 5). If the uptake and/or depuration curve is obviously not first order, then more complex models should be employed (*cf.* references in Annex 5).

H. azteca weight data

57. *H. azteca* wet weights should be determined individually for each of the (triplicate) pooled samples taken at each sampling interval for test (and control) groups during the uptake and the depuration phases. The dry mass should also be reported to allow conversion of lipid concentration from a wet to a dry mass basis. Care should be taken that a suitable microbalance is used to determine the small mass of the test organisms

Kinetic and steady-state bioconcentration factors

58. Kinetic and steady-state bioconcentration factors should also be reported relative to a default tissue lipid content of 3% (w/w), unless there is evidence that the test chemical does not primarily accumulate in lipid. *H. azteca* tissue concentration data, or the BCF, are normalised according to the ratio between 3% (*cf.* paragraph 13) and the actual (individual) mean lipid content (in % wet weight) (*cf.* Annex 5). Lipid normalization of BCF_K and BCF_{SS} values is required to improve the comparability of the results from different *H. azteca* bioconcentration tests.

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Interpretation of results

59. The results should be interpreted with caution where measured concentrations of test solutions occur at levels near the detection limit of quantification of the analytical method.

60. Clearly defined uptake and depuration curves are an indication of good quality bioconcentration data. For the rate constants, the result of a χ^2 goodness-of-fit-test should show a good fit (i.e. small measurement error percentage (27)) for the bioaccumulation model, so that the rate constants can be considered reliable (*cf.* Annex 5).

61. If two or more concentrations are tested, the results of both or all concentrations are used to examine whether the results are consistent and to show whether there is a concentration dependence of the BCF.

62. The resulting BCF_{SS} is questionable if the BCF_K is significantly larger, as this can be an indication that steady-state has not been reached or loss processes have not been taken into account. In cases where the BCF_{SS} is very much higher than the BCF_K, the derivation of the uptake and depuration rate constants should be checked for errors and re-evaluated. A different fitting procedure might improve the estimate of BCF_K (*cf.* Annex 5).

Test Report

63. The test report should include the following information:

Test chemical

- Physical nature and, where relevant, physicochemical properties;
  - Chemical identification data, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate);
  - For multi-constituent substances and UVCB (chemical substances of Unknown or Variable composition, Complex reaction products and Biological materials) describe as far as possible the chemical identity of the individual constituents and for each its percentage of the total mass of the substance. Summarize how the analytical method used in the test reflects a measure of the concentration of the substance;
  - If radiolabelled, the specific activity, the precise position of the labelled atom(s) and the percentage of radioactivity associated with impurities;
  - Storage conditions of the test chemical and stability of the test chemical under storage conditions if stored prior to use.

Test species

- Scientific name, source, any pre-treatment, acclimation (if necessary), age, sex.

Test conditions

- Test procedure used (e.g. flow-through or semi-static); application method (e.g. stock solutions or passive dosing systems);
- Type and characteristics of illumination used and photoperiod(s);
- Detailed information on husbandry;
- Test design (e.g. number and size of test vessels, water volume replacement rate, loading rate, number of replicates, number of *H. azteca* per replicate, number of test concentrations (if applicable), length of uptake and depuration phases, sampling frequency for *H. azteca* and water samples);
- Method of preparation of stock solutions and frequency of renewal (the solvent, its concentration and its contribution to the organic carbon content of test water should be given, when used) or description of alternative dosing system;
- The nominal test concentration in the test medium, the means of the measured values and their standard deviations in the test vessels and the method by which these were attained (Each data point for the water concentrations should be reported, in order to be able to reconstruct the applied aquatic exposure concentration).
- Source of the dilution water, description of any pre-treatment and water characteristics: pH, hardness, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), conductivity (if measured), total organic carbon, and any other measurements made;

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- Water quality within test vessels, pH, hardness, TOC, temperature and dissolved oxygen concentration including methods used and frequency of measurements;
- Detailed information on feeding, e.g. type of food(s), source, composition (at least lipid and protein content if possible), amount given and frequency as well as cleaning of the test vessels.
- Information on the treatment of *H. azteca* and water samples, including details of preparation, storage, extraction and analytical procedures (and precision) for the test chemical and lipid content.
- Date of introduction of test organisms to test solutions and test duration.

Results

- Results from any preliminary work including toxicity investigations;
- Mortality of the *H. azteca* during the study and any observed abnormal behaviour;
- Information on any adverse effects observed (reduced movement, colour change, water turbidity, …);
- Complete description of all analytical procedures employed including limits of detection and quantification, variability and recovery;
- The lipid content of the *H. azteca*, including the method used, and if derived lipid normalisation factor (Ln, factor to express results relative to a tissue lipid content of 3%);
- Tabulated *H. azteca* wet weight data, linked to each sample's chemical concentrations (and lipid content, if applicable), both for control and exposure groups (for example using unique identifiers for each sample);
- Tabulated test chemical concentration data in *H. azteca* (C_H, linked to each individual sample) and water (C_w) (with TWA values for test group, standard deviation and range, if appropriate) for each sampling time (C_H expressed in mg/kg wet weight of whole body or specified tissues thereof e.g. lipid, and C_w in mg/L). C_w values for the control series (background should also be reported);
- Plots (including all measured data), showing the following (if applicable, concentrations may be expressed in relation to the whole body and the lipid content normalised to 3% of the amphipods):

  - the uptake and depuration of the test chemical in *H. azteca*

  - the time to steady-state (if achieved)

  - both uptake and depuration phase curves, showing both the data and the fitted model

- If a visual inspection of a plot shows obvious outliers, a statistically valid outlier test may be applied to remove spurious data points as well as documented justification for their omission;
- The steady-state bioconcentration factor, (BCF_{SS}), if steady-state is achieved;
- The kinetic bioconcentration factor (BCF_K) and derived uptake and depuration rate constants k_1 and k_2, together with the variances/errors in k_2 (slope and intercept if sequential fitting is used);
- Confidence limits, standard deviation (as available) and methods of computation/data analysis for each parameter for each concentration of test chemical used;
- Any information concerning metabolites and their accumulation;
- Anything unusual about the test, any deviation from these procedures, and any other relevant information;
- A summary table of relevant measured and calculated data, as exemplified below:

Table 1: Exemplary table to list uptake and depuration rate constants and bioconcentration factors of a HYBIT study

k_1 (overall uptake rate constant; L kg^{-1} day^{-1})	Insert Value (95% CI)(1)
k_2 (overall depuration rate constant; day^{-1})	Insert Value (95% CI)(1)
C_H (chemical concentration in *H. azteca* at steady-state(1); mg kg^{-1})	Insert Value ± SD(2)
C_w (chemical concentration in the water; mg L^{-1})	Insert Value ± SD(2)
Ln (lipid normalisation factor)	Insert Value ± SD(2)
BCF_{ss} (steady-state BCF; L kg^{-1})	Insert Value ± SD(2)
BCF_{SSL} (lipid normalised steady-state BCF; L kg^{-1})	Insert Value ± SD(2)
BCF_K (kinetic BCF; L kg^{-1})	Insert Value (95% CI)(1)
BCF_{KL} (lipid normalised kinetic BCF; L kg^{-1})	Insert Value (95% CI)(1)

(1) CI: confidence interval (where possible to estimate)

(2) SD: Standard deviation (where possible to estimate)

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64. Results reported as "not detected/quantified at the limit of detection/quantification" by pre-test method development and experimental design should be avoided, since such results cannot be used for rate constant calculations.

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ANNEX 1: DEFINITIONS AND UNITS

BCF: The bioconcentration factor (BCF) at any time during the uptake phase of this accumulation test is the concentration of test chemical in/on *H. azteca* (C_H as mg/kg) divided by the concentration of the chemical in the surrounding medium (C_w as mg/L). BCF is expressed in $L \cdot kg^{-1}$. Please note that corrections for a standard lipid content are not accounted for.

BCF_K: The kinetic bioconcentration factor (BCF_K) is the ratio of the uptake rate constant, k_1, to the depuration rate constant, k_2 (i.e. k_1/k_2 – *cf*. respective definitions). In principle the value should be comparable to the BCF_{SS} (*cf*. respective definition), but deviations may occur if steady-state was uncertain.

BCF_{KL}: The lipid normalised kinetic bioconcentration factor (BCF_{KL}) is normalised to *H. azteca* tissue with a 3% lipid content.

BCF_{SS}: The steady-state bioconcentration factor (BCF_{SS}) does not change significantly over a prolonged period of time, the concentration of the test chemical in the surrounding medium being constant during this period of time (*cf*. definition of steady-state).

BCF_{SSL}: The lipid normalised steady-state bioconcentration factor (BCF_{SSL}) is normalised to *H. azteca* tissue with a 3% lipid content.

Bioaccumulation: Bioaccumulation is generally referred to as a process in which the chemical concentration in an organism achieves a level that exceeds that in the respiratory medium (e.g., water for a fish or air for a mammal), the diet, or both (1).

Bioconcentration: Bioconcentration is the increase in concentration of the test chemical in or on an organism (or specified tissues thereof) relative to the concentration of test chemical in the surrounding medium.

Depuration: The depuration or post-exposure (loss) phase is the time, following the transfer of the test *H. azteca* from a medium containing test chemical to a medium free of that chemical, during which the depuration (or the net loss) of the chemical from the test *H. azteca* (or specified tissue thereof) is studied.

DOC: Dissolved organic carbon (DOC) is a measure of the concentration of carbon originating from dissolved organic sources in the test media.

Exposure phase: *cf.* 'Uptake phase'.

HCB: Hexachlorobenzene is an organochloride with the molecular formula C_6Cl_6.

k_1: The uptake rate constant (k_1) is the numerical value defining the rate of increase in the concentration of test chemical in/on test *H. azteca* (or specified tissues thereof) when the *H. azteca* are exposed to that chemical (k_1 is expressed in L kg^{-1} day^{-1}).

k_2: The depuration (loss) rate constant (k_2) is the numerical value defining the rate of reduction in the concentration of the test chemical in the test *H. azteca* (or specified tissues thereof) following the transfer of the test *H. azteca* from a medium containing the test chemical to a medium free of that chemical (k_2 is expressed in day^{-1}).

K_{OW}: The octanol-water partition coefficient (K_{OW}) is the ratio of a chemical's solubility in n-octanol and water at equilibrium (OECD Guidelines 107 (2), 117 (3), 123 (4)); also expressed as P_{OW}. The logarithm of K_{OW} is used as an indication of a chemical's potential for bioconcentration by aquatic organisms.

LC_{50}: Lethal Concentration 50. The exposure concentration of a toxic substance lethal to half of the test animals.

LOQ: limit of quantitation

MN: Manufactured nanomaterials.

NOEC: No observed effect concentration

SD: Standard deviation

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SPME: Solid-phase microextraction (SPME) is a solvent-free analytical technique developed for dilute systems. In this method, a polymercoated fiber is exposed to the gas or liquid phase containing the analyte of interest. Generally, a minimum analysis time is imposed so that equilibrium conditions are established between the solid and fluid phases, with respect to the measured species. Subsequently the concentration of the analyte of interest can be determined directly from the fiber or after extracting it from the fiber into a solvent, depending on the determination technique.

Steady-state: A steady-state is reached in the plot of test chemical in *H. azteca* (C_H) against time when the curve becomes parallel to the time axis and three successive analyses of C_H within an appropriate time spacing are within ± 20% of each other, and there are no significant increases among the three sampling periods. When pooled samples are analysed at least three successive analyses are required. For test chemicals which are taken up slowly the intervals need to be adjusted accordingly.

TOC: Total organic carbon (TOC) is a measure of the concentration of carbon originating from all organic sources in the test media, including particulate and dissolved sources.

TRR: Total radioactive residues.

TWA: time-weighted average (water concentration)

Uptake phase: The exposure or uptake phase is the time during which the *H. azteca* are exposed to the test chemical.

UVCB: Chemical substances of unknown or variable composition, complex reaction products and biological materials.

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ANNEX 2: PROPOSAL FOR A LABORATORY HUSBANDRY METHOD FOR *HYALELLA AZTECA*

The suggested procedure for the laboratory husbandry of *H. azteca* is based on the protocol of Borgmann, 1996 (1). During husbandry, the amphipods (15 males and 15 females) are kept in culture medium in 2 L beakers (e.g. polypropylene). The culture medium is based on an established method (1) and contains the essential mineral nutrients (Table A2-1). For the preparation, 500-fold concentrated stock solutions of the minerals (solution 1-3, *cf*. Table A2-1) are used. The 2 L beakers are filled up with holding and dilution water or deionized water and the three solutions are added (each 4 ml).

Table A2-1: Culture medium *H. azteca* (1)

		Culture medium [mM]	Stock solution [mM]	Stock solution [g/L]
Solution 1	$CaCl_2$	1	500	73.51
	NaBr	0.01	5	0.5145
	KCl	0.05	25	1.864
Solution 2	$NaHCO_3$	1	500	42.0
Solution 3	$MgSO_4$	0.25	125	30.81

The use of other culture media (e.g. Elendt M4) for laboratory husbandry of *H. azteca* is possible as long as a comparable reproduction and health status of the animals is ensured. A detailed description of the culture conditions needs to be included in the test report.

General holding conditions:

Table A 2-2: Temperature, aeration and light conditions during *H. azteca* husbandry

Water temperature	23 ± 2°C
Aeration	No additional aeration during husbandry
Illuminance	500 to 1000 lux
Light quality	Wide-spectrum fluorescent lights, 840 K
Photoperiod	16 h light : 8 h dark
Refugium for amphipods	Mesh of cotton gauze or nylon mesh (5 x 5 cm slices)
Feeding	Two times per week with ground fish food flakes; once per week with 4:1 ground fish food flakes:spirulina powder

H. azteca are fed with commercial fish flakes, which have been ground to fine powder using a porcelain mortar or similar. Feeding is carried out 2 times per week by adding 20 - 30 mg of the fish flakes powder to each of the beakers. Once per week the same amount containing a mixture of 4:1 ground fish flakes and spirulina powder is added to each beaker. To dip the food under the water surface, it is sprayed with holding and dilution water by using a manual pump spray or similar. In addition, every beaker contains an approximately 5 x 5 cm piece of gauze which serves as place of refuge. Since the gauze is gradually consumed by *H. azteca*, the availability should be checked weekly and the gauze replaced if needed. For the establishment of a new laboratory husbandry, at least 150 – 200 adult amphipods are needed. These amphipods are subdivided to at least 5 beakers and form the stock culture.

Each beaker contains 15 male and 15 female *H. azteca* each, which are sieved weekly with two Artemia sieves (900 µm and 180 µm) to separate the juvenile amphipods. The juveniles collected from 5 beakers form a new group of offspring which is placed in a 2 L beaker containing culture medium. *H. azteca* are fed with ground commercial fish flakes following the same protocol as applied for maintaining the stock culture. When the offspring reach the age of 7-9 weeks, new groups of stock culture (containing 15 male and 15 female *H. azteca*) can be formed until at least 20 groups (beakers) are installed, which are required to obtain a constant supply of *H. azteca* for bioconcentration studies. The number of adult amphipods per beaker of the stock culture is checked monthly. Missing amphipods should be replaced by young adult amphipods, male or female as required, taken from the offspring (at least 2 months old).

In the stock culture, culture medium is replaced on a weekly basis, when juvenile and adult amphipods are separated by sieving. The groups of offspring are sieved (900 µm and 180 µm) once every three to four weeks during media replacement to remove juvenile amphipods. The formation of a moderate biofilm in the beakers

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has a positive effect on the culture conditions and beakers should thus be used without cleaning for about a month.

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ANNEX 3: INFORMATION ON ACCEPTABLE DILUTION AND TEST WATER

The culture medium described in Borgmann, 1996 (1) should be used for a prolonged *H. azteca* culture. For bioconcentration tests, a medium switch can be performed if no adverse effects occur after the medium switch.

The following water types were successfully applied as dilution water in bioconcentration studies with *H. azteca*:

- De-chlorinated, copper reduced, aerated tap water
- Borgmann medium (1) (*cf.* Annex 2)
- Reconstituted water, e.g., according to EN ISO 6341 (including NaBr)
- Elendt M4 medium (including NaBr)

Certain water quality parameters should not be exceeded, similarly to the OECD TG 305 (2), as listed in Table A3-1 (to be monitored for natural water and tapwater).

Table A3-1: Recommended maximum concentrations of selected water parameters as given in OECD TG 305 (2)

Substance	Limit concentration
Particulate matter	5 mg/L
Total organic carbon	2 mg/L
Un-ionised 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
Aluminium	1 µg/L
Arsenic	1 µg/L
Chromium	1 µg/L
Cobalt	1 µg/L
Copper	1 µg/L
Iron	1 µg/L
Lead	1 µg/L
Nickel	1 µg/L
Zinc	1 µg/L
Cadmium	100 ng/L
Mercury	100 ng/L
Silver	100 ng/L

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ANNEX 4: EXEMPLARY SAMPLING SCHEDULE

Table A4-1: Exemplary sampling schedule for a semi-static exposure test

	Hours	***H. azteca* samples, tissue analysis**	***H. azteca* samples, lipid analysis**	**Test medium samples (fresh)***	**Test medium samples (aged)***
Uptake phase	0	3 x 20 *H.a.***	3 x 10 *H.a.***	2 x 10 mL	
	1	3 x 20 *H.a.*			
	3	3 x 20 *H.a.*			
	6	3 x 20 *H.a.*			
	24	3 x 20 *H.a.*		2 x 10 mL	2 x 10 mL
	48	3 x 20 *H.a.*		2 x 10 mL	2 x 10 mL
	72	3 x 20 *H.a.*	3 x 10 *H.a.*		2 x 10 mL
Depuration phase	1 (73)	3 x 20 *H.a.*		2 x 10 mL	
	3 (75)	3 x 20 *H.a.*			
	6 (78)	3 x 20 *H.a.*			
	24 (96)	3 x 20 *H.a.*		2 x 10 mL	2 x 10 mL
	48 (120)	3 x 20 *H.a.*		2 x 10 mL	2 x 10 mL
	72 (144)	3 x 20 *H.a.*	3 x 10 *H.a.*		2 x 10 mL
Summary:		13 x 60 *H.a.* = 780 *H.a.* 780 *H.a.* + 90 *H.a.* = 870 *H.a.* 870 *H.a.* x 1.2*** = **1044 *H.a.***	3 x 30 *H.a.* = 90 *H.a.*	12 x 10 mL	12 x 10 mL

*Water concentration is checked in the aged and fresh medium prior to and after medium exchange, respectively.

** *H. azteca* at t=0 are collected from the batch of male amphipods just before the amphipods are placed in the test vessel.

***further amphipods (approx. 20%) should be added when stocking the test vessels to compensate potential losses.

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Table A4-2*:* Exemplary sampling schedule for a flow-through exposure test

	Hours	*H. azteca* samples, tissue analysis	*H. azteca* samples, lipid analysis	Test medium samples
Uptake phase	0	3 x 20 *H.a.***	3 x 10 *H.a.***	2 x 10 mL
	1	3 x 20 *H.a.*		
	3	3 x 20 *H.a.*		
	6	3 x 20 *H.a.*		
	24	3 x 20 *H.a.*		2 x 10 mL
	48	3 x 20 *H.a.*		2 x 10 mL
	72	3 x 20 *H.a.*	3 x 10 *H.a.*	2 x 10 mL
Depuration phase	1 (73)	3 x 20 *H.a.*		2 x 10 mL
	3 (75)	3 x 20 *H.a.*		
	6 (78)	3 x 20 *H.a.*		
	24 (96)	3 x 20 *H.a.*		2 x 10 mL
	48 (120)	3 x 20 *H.a.*		2 x 10 mL
	72 (144)	3 x 20 *H.a.*	3 x 10 *H.a.*	2 x 10 mL
Summary		13 x 60 *H.a.* = 780 *H.a.* 780 *H.a.* + 90 *H.a.* = 870 *H.a.* 870 *H.a.* x 1.2** = **1044 *H.a.***	3 x 30 *H.a.* = 90 *H.a.*	16 x 10 mL

* *H. azteca* at t=0 are collected from the batch of male amphipods just before amphipods are placed in the test vessel.

**further amphipods (approx. 20%) should be added to compensate potential losses.

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ANNEX 5: CALCULATIONS

The bioconcentration factor (BCF) is determined based on the measured test item concentrations in *H. azteca* and in water samples collected during the uptake phase as well as during the depuration phase of the study. The method used for BCF determination in *H. azteca* is largely based on the method described for fish in Annex 5 of the OECD test guideline 305 (1). Detailed assumptions for the applied bioconcentration model can be found there. In contrast to the BCF determination in fish according to OECD TG 305, growth can be neglected in *H. azteca* BCF calculation due to the short duration of the studies and the use of adult *H. azteca* in the tests.

In a standard BCF test uptake and depuration can be described in terms of two first order kinetic processes:

$$Rate\ of\ uptake = k_1 * C_w \quad \text{[Equation A5.1]}$$

$$Overall\ depuration\ rate = k_2 * C_H \quad \text{[Equation A5.2]}$$

At steady-state, assuming growth and metabolism are negligible, the rate of uptake equals the rate of depuration, and so combining Equation A5.1 and Equation A5.2 gives the following relationship:

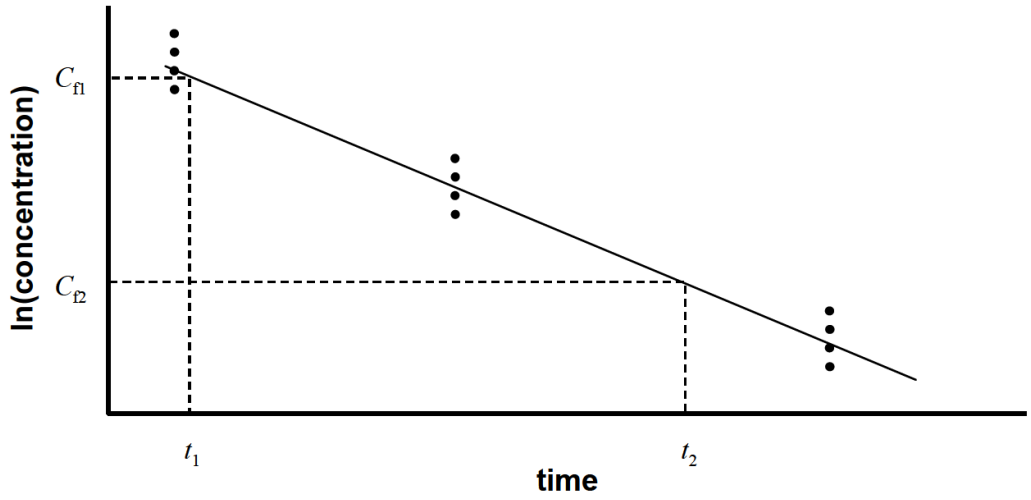
$$BCF = \frac{C_{H\,@\,SS}}{C_{w\,@\,SS}} = \frac{k_1}{k_2} \quad \text{[Equation A5.3]}$$

Where
$C_{H\,@\,SS}$ = Concentration in *H. azteca* tissue at steady-state (mg kg^{-1} wet weight).
$C_{w\,@\,SS}$ = Concentration in water at steady-state (mg L^{-1}).

The ratio of k_1/k_2 is known as the kinetic BCF (BCF_K) and should be equal to the steady-state BCF (BCF_{SS}) obtained from the ratio of the steady-state concentration in *H. azteca* tissue to that in water, but deviations may occur if steady-state was uncertain. However, as k_1 and k_2 are constants, steady-state does not need to be reached to derive a BCF_K.

Sequential method: determination of depuration (loss) rate constant k_2

Most bioconcentration data have been assumed to be "reasonably" well described by a simple two-compartment/two-parameter model, as indicated by the rectilinear curve which approximates to the points for concentrations in Hyalella (on a natural logarithmic (ln) scale), during the depuration phase.



The graphical method may be applied for resolving types of depuration deviating from first order kinetics. Note that deviations from a straight line may indicate a more complex depuration pattern than first order kinetics. Such deviations are often observed if total radioactivity (TRR) is used as basis for the calculations.

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To calculate k_2 for multiple time (sampling) points, perform a linear regression of logarithmized (ln) concentrations versus time. The slope of the regression line is an estimate of the depuration rate constant k_2 (2). From the intercept the average concentration in the *H. azteca* at the start of the depuration phase ($C_{0,d}$; which equals the average concentration in *H. azteca* tissue at the end of the uptake phase) can easily be calculated (including error margins) (2).

$$C_{0,d} = e^{intercept}$$ [Equation A5.4]

Furthermore, depuration (loss) rate constant k_2 can be calculated using the following equation:

$$C_{H,depuration}(t) = C_{H,end\ uptake} * e^{-k2\,t}$$ [Equation A5.5]

where

$C_{H,depuration}(t)$

is the concentration in Hyalella at the time t of the depuration phase, and

$C_{H,end\ uptake}$

is the average concentration in Hyalella at the end of the uptake phase.

Sequential method: determination of uptake rate constant k_1

To find a value for k1 given a set of sequential time concentration data for the uptake phase, use a computer programme to fit the following model:

$$C_H(t) = C_w(t) * \frac{k_1}{k_2} * (1 - e^{-k_2 * t})$$ [Equation A5.6]

Where k_2 is given by the previous calculation, $C_H(t)$ and $C_w(t)$ are the concentrations in *H. azteca* tissue and water, respectively, at time t.

The sequential method should be considered as less reliable, if there is a significant difference between $C_{0,d}$ determined from the depuration phase and $C_{H(t)}$ determined as average tissue concentration at the end of the uptake phase. A clear gap indicates that the fitting does not lead to a satisfactory result.

Although the test was developed for organic chemicals, it could also be used for metals. In this case any concentration of the tested chemical that is already detectable in the organism before being exposed should be considered. This is relevant since some metals are present in the organism as essential metals at physiological levels:

$$C_H(t) = C_H(t0) + C_w(t) * \frac{k_1}{k_2} * (1 - e^{-k_2 * t})$$ [Equation A5.7]

where

$C_H\ (t0)$ = concentration of test chemical in Hyalella before being exposed

Simultaneous method: determination of uptake and depuration rate constants

Simultaneous method for the calculation of uptake and depuration (loss) rate constants. Computer programmes can be used to find values for k_1 and k_2 given a set of sequential time concentration data and the model:

$$C_H = C_w * \frac{k_1}{k_2} * \left(1 - e^{-k_2 * t}\right) \qquad 0 < t < t_c$$ [Equation A5.8]

$$C_H = C_w * \frac{k_1}{k_2} * (e^{-k_2\,(t - t_c)} - e^{-k_2 * t}) \qquad t > t_c$$ [Equation A5.9]

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where

t_c = time at the end of the uptake phase

This approach directly provides standard errors for the estimates of k_1 and k_2. When k_1/k_2 is substituted by BCF (*cf.* Equation A5.3) in Equation A5.6 and Equation A5.7, the standard error and 95% CI of the BCF can be estimated as well. This is especially useful when comparing different estimates due to data transformation. The dependent variable (*H. azteca* tissue concentration) can be fitted with or without ln transformation, and the resulting BCF uncertainty can be evaluated.

The guidance document to OECD TG 305 (3) proposes a package for the programming software R that enables such an estimation. Information on the application of the R-package and interpretation of the calculated results can be obtained from the OECD web page (https://www.oecd.org/chemicalsafety/testing/section-3-environmental-fate-behaviour-software-tg-305.htm link valid June 2023).

Calculation of the TWA (flow-through exposure)

In the case water samples were drawn in two or more unequal time intervals, time-weighted average (TWA) concentrations of the test chemical in the test solutions should be determined to account for the variation in time between samplings (i.e. accounting for the time interval represented by a pair of samples. To this end, weighted average concentrations are calculated by multiplying the average of two subsequently measured concentrations by the time period (h) that elapsed between both measurements.

Afterwards all weighted average concentrations are then summed up and divided by the total time (h) of the uptake period resulting in the TWA concentration using the following equation described in OECD GD to OECD TG 305 (3):

$$C_w = \frac{\sum_{i=1}^{n} \frac{\left(C_{start,i} + C_{end,i}\right)}{2} w_i}{\sum_{i=1}^{n} w_i}$$

[Equation A5.10]

Where:

C_w is the time-weighted average concentration

n is the number of sampling periods

$C_{start,i}$ is the concentration of the fresh test solution of period i

$C_{end,i}$ is the concentration of the old solution of period i

w_i is time $ti - ti\text{-}1$, the number of hours or days in the interval between measurements of concentration

Calculation of the TWA (semi-static exposure)

In case of a semi-static exposure scenario, the concentration of the test chemical can decline over the period between medium renewals. A TWA for this scenario can be calculated according to the method described in Annex 6 of the OECD TG 211 (4). The following equation is applied for the measured concentrations at start and end of a given sampling interval:

$$Area = \frac{Conc_0 - Conc_1}{ln(Conc_0) - ln\,(Conc_1)}\ x\ days$$

[Equation A5.11]

Where:

$Conc_0$ is the measured concentration at the start of a given renewal interval

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902 $Conc_1$ is the measured concentration at the end of given renewal interval

903 ln ($Conc_0$) is the natural logarithm of $Conc_0$

904 ln ($Conc_1$) is the natural logarithm of $Conc_1$

905 Days is the number of days in the renewal interval

906 Area is the area under the exponential curve for a given renewal interval

907 The time-weighted average (TWA) is the sum of all areas ("Total Area") divided by the sum of all days in all
908 renewal intervals.

909

910 **References:**

911 1. OECD (2012), Test No. 305: Bioaccumulation in Fish: Aqueous and Dietary Exposure, OECD
912 Guidelines for the Testing of Chemicals, Section 3, OECD Publishing, Paris,
913 https://doi.org/10.1787/9789264185296-en.
914 2. Kristensen, P, Nyholm, N (1987), Bioaccumulation of chemical substances in fish: the flow-through
915 method - Ring Test Programme, 1984-1985 Final report, CEC, March 1987.
916 3. OECD (2017), Guidance Document on Aspects of OECD TG 305 on Fish Bioaccumulation, OECD
917 Environment, Health and Safety Publications, Series on Testing & Assessment No. 264,
918 ENV/JM/MONO(2017)16, 19-Jul-2017.
919 4. OECD (2012), Test No. 211: *Daphnia magna* Reproduction Test, OECD Guidelines for the Testing of
920 Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264185203-en.).

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ANNEX 6: LIPID DETERMINATION IN *H. AZTECA*

For determination of the lipid content of the test organisms (e.g. 10 animals per pooled sample), the lipid extraction method of Smedes (1) adapted by Schlechtriem et al. (2) should be used. Note that with decreasing sample mass increased standard deviations for lipid measurements were observed in several cases (OECD validation report, in prep.). If a lower sample mass is measured the number of pooled animals per sample should be increased to reach a sufficient sample mass (> 50mg/pooled sample).

Material needed:

- Small glass vials (7 ml, one glass per sample, e.g. liquid scintillation vails)
- drying cabinet
- desiccator
- centrifuge
- glass test tubes (at least 10 ml)
- homogeniser with Teflon pestle
- Vortex generator
- Pasteur pipette
- Cyclohexane
- Isopropanol
- N_2 for evaporating

The small glass vials are stored over night at 75°C in a drying cabinet, placed in a desiccator for additional 30 min and weighted (empty). They are used to pool the lipid extract.

The amphipods, the fresh weight of which was determined immediately after sampling, are transferred into glass test tubes. After 200 µl of solution 1 (Table A6-1) are added to the tube, the amphipods are homogenised for about 1 min with a homogeniser with Teflon pestle. The pestle is rinsed with 4.3 ml solution 1, which are also collected in the tube. After 2.75 ml of distilled water are added, the tube is vortexed and centrifuged (12 min, Relative Centrifugal Force approx. 500 x g). The organic phase is transferred into the small glass vial using a Pasteur pipette.

After 2.5 ml of solution 2 (Table A6-1) have been added to the remaining aqueous phase, the tube is vortexed again and centrifuged under the same conditions. The organic phase is pooled with the first one and evaporated with nitrogen until only the lipid phase is left. The extract in the glass vial is stored over night at 75°C in a drying cabinet, placed in a desiccator for additional 30 min and weighted again. The net dry weight is determined with a microbalance (precision 0.001 mg) for a total lipid content by weight.

Table A6-1: Working solutions for lipid extraction

	Composition
Solution 1	Cyclohexane / Isopropanol 5:4 (v/v)
Solution 2	Cyclohexane / Isopropanol 87:13 (v/v)

References:

1. Smedes F (1999), Determination of total lipid using non-chlorinated solvents. Analyst 124(11):1711–8. Available from: http://xlink.rsc.org/?DOI=a905904k

2. Schlechtriem C, Fliedner A, Schäfers C (2012), Determination of lipid content in fish samples from bioaccumulation studies: Contributions to the revision of guideline OECD 305. Environ Sci Eur 24(4):1 7. Available from: https://doi.org/10.1186/2190-4715-24-13

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ANNEX 7: SEXING OF *H. AZTECA*

To collect male or female amphipods, adult *H. azteca* (older than 2 months) are transferred into a petri dish and examined under a stereomicroscope (magnification factor: 6 – 10 x). Eggs are visible in the marsupium at the ventral side of the female. The specific sexual characteristics of male *H. azteca* are gnathopods located on the front body (Figure A7-1).

During the mating process, the male amphipod is attached to the dorsal side of the female amphipod. Female and male amphipods can be separated by using spring steel tweezers. Generally, only healthy amphipods are selected. Test organisms which are used in bioconcentration studies should be about 8 weeks old but not older than 6 months when selected for a test. An Artemia sieve of wider mesh size (900 µm) is used to separate larger amphipods and to obtain test organisms of similar size. The male amphipods are collected, counted, and transferred into beakers (2 L polypropylene) filled with a mix of culture medium (50%) and holding and dilution water (50%) to allow gradual adaptation of the amphipods to the test water. Instead of the cotton gauze used during husbandry, steel mesh shelters are used during the study and are placed in the beakers to provide a sufficiently dimensioned place of refuge for the dense group of *H. azteca*. However, each beaker should not contain more than 130 to 150 amphipods to avoid competitive behaviour and cannibalism. The selected test organisms will remain in the collection beakers until the start of the test. The holding conditions (feeding, light, temperature) during this phase are in agreement with the husbandry conditions described above (*cf.* Annex 2). The sexing should take place 1 - 2 days before test start. If the time period between separating and test start is longer than 2 days, the amount of selected male amphipods should be re-counted. If necessary, amphipod losses should be replaced by additional male amphipods. Materials required for amphipod sexing are described in Table A7-1.

Figure A7-1: Sexual dimorphism in *H. azteca*. Arrows indicate sex-specific characteristics. A: female *H. azteca* with eggs; B: male *H. azteca* with gnathopods

MATERIAL LIST - SEXING

Table A7-1: Compilation of materials needed for *H. azteca* sexing

Article	Remarks
Stereomicroscope	
Spring steel tweezer	
Tip-cut plastic Pasteur pipets	
Mesh of stainless steel	three parts per 20 L test aquarium required
2 L polypropylen beaker	
Food TetraMin® (ground fish food flakes)	
Petri dishes	

ANNEX 8: PREPARATION OF TEST FOOD (DECOTAB)

A pre-test investigating the suitability of different food items was conducted to find the most appropriate feeding protocol for *H. azteca* in BCF studies (1). In this study, the filter disc method (2) was compared to agar-bound feed, so called Decotabs, which were prepared according to Kampfraath et al. (2012) (3). Decotab feeding is the recommended feeding method for HYBIT tests because feeding the agar-agar cubes enriched with ground fish food flakes ensures optimal nutrient supply to the amphipods while algal growth in the test system remains low. Decotabs are readily accepted by *H. azteca*.

The preparation of the Decotabs should be done as follows:

Material:

- A silicone tray with wells that provide a volume of approx. 1 mL (here we use a cubic shape)
- 1 mL 2% agar solution per cube
- 75 mg finely ground fish food per cube

An appropriate volume of a 2% agar-agar solution in ultra-pure H_2O solution is boiled on a heated plate under stirring until the agar-agar has dissolved completely. After a short cool-down phase, ground fish food flakes are added to the solution equivalent to 75 mg ground fish food per mL. The suspension is stirred and poured into the wells of the silicone tray. The agar-agar cubes solidify rapidly. The silicone tray is then sealed with a plastic bag to avoid evaporation and stored at 4°C. The cubes will start to deteriorate after 8-10 days and should therefore be used within 7 days. Alternatively, the cubes can be stored at -20°C for a prolonged time (approx. 2 - 4 weeks, should be evaluated by each lab). Prior to feeding, the frozen cubes should have been thawed thoroughly.

References:

1. Kosfeld V, Fu Q, Ebersbach I, Esser D, Schauerte A, Bischof I, Hollender J, Schlechtriem C (2020), Comparison of alternative methods for bioaccumulation assessment: Scope and limitations of in vitro depletion assays with rainbow trout and bioconcentration tests in the freshwater amphipod *Hyalella azteca*. Environ Toxicol Chem 39(9):1813–1825. Available from: DOI: 10.1002/etc.4791.

2. Schlechtriem C, Kampe S, Bruckert HJ, Bischof I, Ebersbach I, Kosfeld V, Kotthoff M, Schäfers C, L'Haridon J (2019), Bioconcentration studies with the freshwater amphipod *Hyalella azteca*: are the results predictive of bioconcentration in fish? Environ Sci Pollut Res 26:1628–1641. doi.org/10.1007/s11356-018-3677-4.

3. Kampfraath AA, Hunting ER, Mulder C, Breure AM, Gessner MO, Kraak MHS, Admiraal W (2012), DECOTAB: a multipurpose standard substrate to assess effects of litter quality on microbial decomposition and invertebrate consumption. Freshw Sci 31(4):1156–62. Available from: http://www.journals.uchicago.edu/doi/10.1899/12-075.1.

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ANNEX 9: PROPOSAL FOR A TOXICITY TEST AS PRELIMINARY EXPERIMENT FOR HYBIT TESTS

Since toxic effects are not desired and should be avoided in bioconcentration studies (1), it is important to select an exposure concentration that does not cause adverse effects in the test species.
Sufficient information on the toxicity of the test chemical to aquatic invertebrates and/or *H. azteca* is not always available. Data on acute and/or chronic toxicity may be available for the widely tested Daphnia species although this may differ for Hyalella species.
Therefore, an appropriate exposure concentration has to be determined prior to the bioconcentration test in this case. The following paragraphs describe a proposal for such an evaluation in the style of an acute toxicity test with the endpoint mortality.
A semi-static exposure scenario is proposed. However, if the chemical characteristics do not allow for a semi-static exposure, the test setup may have to be changed to a flow-through one. A detailed description of the preliminary investigations should be available in the final study report to justify the selection and relevance of the final treatment level used in the bioconcentration test.

Material:

- Glass aquarium (as water bath)
- Beaker (250 mL)
- Water heating element / climate chamber
- Shortened stainless-steel mesh shelters
- DECOTABs
- Artemia sieves
- Adult *H. azteca* (> 2 months old; male, female or mixed; Amphipods should not be older than 6 months at the start of a test)

Test setup:

- 1 control
- 5 concentrations (treatments)
- 3 - 6 replicates per control / treatment
- 20 *H. azteca* per replicate
- Exposure duration: Approx. the planned duration of the uptake phase in the bioconcentration test (here: 4 days / 96 hrs)
- Exposure method: semi-static (change to flow-through, if necessary / desirable)
- Recommended water temperature of 23°C (± 2°C)
- Daily medium renewal
- Daily temperature and O_2 saturation as well as pH determination
- Randomised placement of beakers in water bath
- Daily feeding with DECOTABs, ¼ cube per day per beaker
- Daily determination of water concentration (fresh and aged medium)
- Daily count of alive and, if visible, dead *H. azteca,* in each beaker

Table A9-1: Exemplary concentrations for a toxicity range-finder test for prochloraz with *H. azteca*. The concentration selection was based on a *G. pulex* LC_{50} (96 hrs) of 2.2 mg/L (2) and the exposure concentration of 50 µg/L in already conducted *H. azteca* bioconcentration tests (3). A spacing of approx. 3.5 was used between all concentrations.

Scenario	Prochloraz in medium (mg/L)
Concentration 1	2.143
Concentration 2	0.612
Concentration 3	0.175
Concentration 4	0.050
Concentration 5	0.014
Control	0.000

Pooling option:

With 36 beakers in the test, daily media renewal and a determination of fresh and aged media concentrations of the test chemical, a considerable number of samples is generated. 'Sample pooling' can help to reduce the number of samples for analyses. Aliquots (5 mL) collected from each beaker (total of 30 mL) should be

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sufficient to determine the average parameters of each treatment. This option should only be selected if there are no indications that the treatments differ significantly from each other.

Validity criteria:

The following validity criteria for a preliminary, acute toxicity test with *H. azteca* may be used:

- Control mortality ≤ 10%
- The water temperature variation should be within the range 23 ± 2°C. However, the temperature should not vary by more than ±1°C within 24 hours;

Troubleshooting:

- Artemia sieves should have no holes / pockets that allow the amphipods to hide in them. Otherwise *H. azteca* loss that is not mortality skews the results.

References:

1. OECD (2012), Test No. 305: Bioaccumulation in Fish: Aqueous and Dietary Exposure, OECD Guidelines for the Testing of Chemicals, Section 3, OECD Publishing, Paris, Available from: https://doi.org/10.1787/9789264185296-en.

2. Beketov MA, Liess M (2008), Potential of 11 pesticides to initiate downstream drift of stream macroinvertebrates. Arch Environ Contam Toxicol 55(2):247–53.

3. Kosfeld V, Fu Q, Ebersbach I, Esser D, Schauerte A, Bischof I, Hollender J, Schlechtriem C (2020), Comparison of alternative methods for bioaccumulation assessment: Scope and limitations of in vitro depletion assays with rainbow trout and bioconcentration tests in the freshwater amphipod *Hyalella azteca*. Environ Toxicol Chem 39(9):1813–1825. Available from: DOI: 10.1002/etc.4791.

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