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

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MULTI-LABORATORY RING-TRIAL TO SUPPORT DEVELOPMENT OF OECD TEST GUIDELINE ON *HYALELLA AZTECA* BIOCONCENTRATION TEST (HYBIT)

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INTRODUCTION

A protocol for carrying out bioconcentration tests with the aquatic invertebrate species *H. azteca* under standardized conditions (HYBIT) was developed as part of project LRI ECO 40 funded by the European Chemical Industry Council (Kosfeld et al. 2020). This protocol includes both a flow-through and a semi-static test design. To support the development of a new OECD test guideline, validation was required to confirm the transferability of the HYBIT test protocol and to prove the reproducibility of the results obtained. For this purpose, an international multilaboratory ring trial has been carried out involving 11 laboratories.

Participating labs could perform bioconcentration studies with *H. azteca* following the semi-static or flow-through design (or both) and were responsible for test performance as well as analyses of chemicals in water and Hyalella samples collected during the bioconcentration studies. Considering all sample replicates and the series of sampling times required to estimate the kinetics of substance uptake and elimination, large test populations of up to 1500 organisms were required. Therefore, guidance on the laboratory husbandry of *H. azteca* was provided.

Populations of adult amphipods consist of male and female animals. However, mixed test groups should be avoided to prevent the reproduction of the animals during BCF studies which would cause elimination of previously accumulated test item by the release of juvenile amphipods. To collect male or female amphipods as test organisms, adult *H. azteca* can be separated according to their specific sexual characteristics. Only male amphipods were used for the bioconcentration studies.

Three chemicals of different properties were tested as part of the ring trial. Test substances were Terbutryn, Prochloraz and Hexachlorobenzene (HCB) which are characterized by low to high hydrophobicity, respectively. Terbutryn and Prochloraz were tested in the semi-static approach. Prochloraz and Hexachlorobenzene were tested in flow-through tests.

Kinetic and steady-state BCF estimates which are known to depend on the lipid content of the test animals were calculated for all test substances (Schlechtriem et al. 2019, Kosfeld et al. 2020). The lipid content in *H. azteca* may vary depending on the size and age of the amphipods and tends to be lower compared to the lipid levels measured in fish used for bioaccumulation testing. Therefore, lipid normalization of the estimated BCF values is required to explain interlaboratory differences in the results obtained during the ring trial and to allow the comparison with BCF estimates from fish studies. Lipid normalization of BCF estimates to a lipid level of 3% (w/w) was carried out which is equivalent to a high lipid content of male field caught amphipods (Arts et al. 1995). However, only the use of suitable extraction techniques guarantees the complete extraction of total lipids from collected test organisms which is required to ensure the correct lipid normalization of BCF values (Schlechtriem et al. 2012). Therefore, a standard protocol for lipid measurements of small size samples is part of the ring test protocol. The correct application of the extraction procedure in the different labs was validated prior to the ring test.

The results of the multi-laboratory ring trial to support the development of a new OECD test guideline on the *H. azteca* bioconcentration test (HYBIT) are described including a comparison of the BCF estimates obtained across the participating laboratories.

MATERIAL AND METHODS

Prior to the ring trial, the HYBIT protocol for *H. azteca* bioconcentration studies (Annex 9) was applied in several studies with Prochloraz, Terbutryn and HCB at the test facilities of UBA, Ineris, and Fraunhofer IME. Studies were carried out under semi-static (Terbutryn, Prochloraz) and flow-through (Prochloraz, HCB) conditions. ^{14}C radiolabeled Prochloraz was applied to assess the suitability of the semi-static approach for radiolabeled studies. The pre-tests confirmed the robustness and transferability of the ring test protocol.

Animal husbandry

Test animals for the bioconcentration tests carried out as part of the ring trial were obtained by laboratory breeding in the participating test facilities (Annex 2). Alternatively, test animals purchased from commercial sources could be used, provided that the animals have been acclimatized appropriately. The procedure applied for the laboratory husbandry of *H. azteca* was based on the protocol of Borgmann and Norwood (2009). During husbandry, the amphipods were kept in culture medium in 2 L polypropylene beakers. The culture medium was based on Borgmann (1996) containing essential mineral nutrients. *H. azteca* were fed with commercial fish flakes TetraMin®, which has been ground to fine powder using a porcelain mortar or similar. Feeding was carried out 3 times per week by adding 20-30 mg of the TetraMin® powder to each of the beakers. In addition, every beaker contained an approximately 5 x 5 cm piece of gauze which served as place of refuge. Since the gauze was consumed by the animals, the availability was checked weekly and the gauze replaced if needed. Each beaker contained 15 male and 15 female *H. azteca each*, which were sieved weekly with two Artemia sieves (900 µm and 180 µm) to separate the juvenile amphipods. Culture medium was replaced on a weekly basis. Water temperature during *H. azteca* husbandry was 25 ± 2°C. No additional aeration was applied. Using wide-spectrum fluorescent lights (840 K) providing a illuminance of 500 to 1000 lux, animals were kept under a 16h light: 8 h dark regime.

Sexing of test animals

Adult *H. azteca* were transferred into a petri dish, examined under a stereomicroscope (magnification factor: 6-10x) and separated based on their specific sexual characteristics. Generally, only male amphipods were selected. Test organisms which were used in the bioconcentration studies were preferred to be older than 2 months. An Artemia sieve of wider mesh size (900 µm) was used to separate larger amphipods and to obtain test organisms of similar size. The male amphipods were collected, counted, and transferred into beakers (2 L polypropylene) filled with a mix of culture medium and holding and dilution water (HDW) (50:50) to allow gradual adaptation of the animals to the test water (HDW) until the start of the test. The holding conditions (feeding, light, temperature) during this phase were in agreement with the husbandry condition described above. The sexing took place 1-2 days before test start.

Test chemical selection

Three chemicals of different properties were tested in the ring trial. Test substances were Terbutryn, Prochloraz and HCB. Table 1 presents the structure, CAS No. and measured log K_{ow} value for each chemical. All substances were previously applied in *Hyalella azteca* bioconcentration tests (Schlechtriem et al. 2019, Kosfeld et al. 2020).

Chemicals and supplies

Terbutryn (99.1% purity) was purchased from Sigma Aldrich (Cat. No. 45677). Prochloraz (>98.6% purity) was obtained from Sigma Aldrich (Cat. No. 45631) as well as HCB (purity > 99%) (Cat. No. 45522).

Ring trial bioconcentration experiments

Eleven laboratories (see Annex 2) were involved in the ring trial, with varying degrees of experience in performing bioconcentration tests, from no experience to having many years of experience in test performance. During the ring trial there were two possible options to

conduct bioconcentration studies with *H. azteca*: using a semi-static test setup with a full daily water exchange or a flow-through approach with an exchange rate of e.g. 5 times the total volume per day. A detailed description of the different test set-ups is provided in Annex 9. For both set-ups holding and dilution water (HDW) fulfilling the requirements defined by OECD 305 (OECD, 2012a) was used to prepare the test media. However, apart from the simple use of HDW further media such as Borgmann medium, Elendt M4 medium, ISO medium or reconstituted medium, were applied (Annex 5). An overview of the bioconcentration experiments conducted by the different participants as part of the ring trial is presented in Table 2.

Preparation of test media

Terbutryn and Prochloraz (semi-static test setup). For the preparation of the basic solution, 750 µL of the acetonic stock solution containing 0.75 mg of test item were pipetted into a 10 L-brown glass bottle with screw cap. After evaporating the solvent to complete dryness, the bottle was filled up to a total volume of 10 L with HDW (or alternative media) to reach a target concentration of 75 µg/L. The basic solution was then stirred overnight (at least 14 h) using a magnetic stirrer. 10 L of the basic solution were added to the aquarium (test chamber), which was then filled with 5 L of HDW (or alternative media) to provide the exposure concentration of 50 µg/L. Finally, the test medium (test solution) was stirred thoroughly to guarantee homogeneous exposure conditions.

Prochloraz (flow-through test setup / solvent-free application). For the preparation of the basic solution, 1 mL of the acetonic stock solution containing 10 mg of test item were pipetted into a 10 L-brown glass bottle with screw cap. After evaporating the solvent to complete dryness, the bottle was filled up to a total volume of 10 L with HDW (or alternative media) to reach a target concentration of 1 mg/L. The basic solution was then stirred overnight (at least 14 h) using a magnetic stirrer. The daily prepared basic solution of the test item (10 L) was

constantly stirred and served as substance reservoir. The reservoir was connected with a membrane pump via a glass capillary tube (PTFE tube fittings). The aqueous solution from the reservoir was pumped at a defined flow rate (5 mL/min) into a mixing chamber with magnetic stirring. Through a second inlet of the mixing chamber HDW (or the alternative medium) was added to reach a defined total flow rate (100 mL/min). The test medium was directed continuously into the experimental tank, which was thermo-regulated by an outer water bath.

HCB (flow-through test setup / solvent-facilitated application). A stock solution of HCB was prepared at a concentration of 1 mg/mL using Dimethylformamide (DMF) as solvent. In total, 20 mL of the stock solution were prepared and stored at ≤8°C. 1 mL of the stock solution was used to prepare an intermediate solution in DMF at a concentration of 10 µg/mL (1:100 dilution). The intermediate solution of HCB was filled into a 50 mL infusion pump syringe (substance reservoir). After connecting the syringe to the infusion pump system, the intermediate solution was pumped at a flow rate of approx. 10 µL/min into a mixing chamber with magnetic stirring. Through a second inlet of the mixing chamber HDW (or an alternative medium) was added to reach a defined total flow rate of approx. 100 mL/min and to provide the exposure concentration of 1 µg/L.

Selection of test concentrations

Toxic effects should be avoided in bioconcentration studies and it is thus important to select exposure concentrations that do not cause adverse effects in the test species over the entire exposure period. However, sufficient information on the toxicity of the three test substances in *H. azteca* were missing and therefore, appropriate exposure concentrations were determined prior to the performance of the bioconcentration tests (Schlechtriem et al. 2019, Kosfeld et al. 2020). All test concentrations applied in the ring trial showed to have no effect on the survival of the animals. Nevertheless, a toxicity test protocol to identify suitable test concentrations was developed involving a semi-static exposure scenario (Annex 3). The

protocol was evaluated prior to the ring-test by three of the participating laboratories using the test substance Prochloraz. The preliminary toxicity tests confirmed that the exposure concentration of 0.05 mg/L that was previously used in Kosfeld et al. (2020) is safe and can be used in the ring test. First toxic effects could be seen only in media concentrations of > 1 mg/L Prochloraz after an exposure time of 96 hrs. For highly hydrophobic substances such as HCB (log K_{ow} 5.86) the semi-static exposure scenario may be inappropriate due to high potential losses caused by adsorption. In this case the preliminary toxicity test can still provide important information on the further testing of the test substance recommending a flow-through application.

Preparation of test food (Decotabs)

Due to the good growth performance in *H. azteca* fed agar–bound flakes (Decotabs) enriched with ground fish food flakes (TetraMin®), Decotab-feeding was the preferred feeding method for the ring test (Kosfeld et al. 2020). Decotabs were prepared according to Kampfraath et al. (2012). In brief, an appropriate volume of a 2% agar solution was boiled in a microwave until the agar has dissolved completely. After a short cool-down phase TetraMin® was added to the solution equivalent to 75 mg ground TetraMin® per mL. The suspension was stirred and poured into the wells of the silicone tray. The agar cubes solidified rapidly. The silicone tray was then sealed with a plastic bag to avoid evaporation and stored at 4°C and were used within 7 days.

Test performance

The HYBIT protocol (Annex 9) includes a semi-static and flow-through test design which largely follow the concept of the aqueous exposure fish test described in OECD TG 305-I (OECD 2012a). As in the fish test, aqueous bioconcentration studies with *H. azteca* are conducted to assess the bioaccumulation potential of chemicals measured by the chemical's bioconcentration factor (BCF). *H. azteca* are exposed to the chemical dissolved in water. *H.*

azteca and water samples are collected and analyzed at certain intervals during the course of the test to ultimately determine uptake and depuration rate constants or bioconcentration factors. The BCF is calculated as the ratio of the concentration in the amphipod to the dissolved concentration in water at "steady-state" (BCF_{SS}), or by the ratio of the uptake and depuration rate constants (BCF_K). An overview over the participating labs and their conducted bioconcentration experiments during the ring test are presented in Table 2. The experimental conditions (temperature, pH, dissolved oxygen) were monitored throughout the bioconcentration tests.

Sampling of *H. azteca* and water

Sampling of *H. azteca* and test medium was carried out according to the schedules presented in Annex 4. The amphipods were removed from the test vessel via a small net. The required number of organisms were transferred with a spring steel tweezer into 50 ml glass beakers filled with water from the test vessels. Each beaker represented one replicate. The remaining organisms were returned into the test chamber. Each replicate was placed in a fine sieve and rinsed in dilution water (approx. 50 ml). After shortly blotted drying with soft paper, the organisms of one replicate were transferred in tared 1.5 ml reaction vials, weighed and immediately frozen at ≤ -18°C. Water samples (10 mL) were sampled in duplicates from the test vessels using a 10 mL pipette (after carefully stirring the water in the test vessels) and instantly added to a glass vial (e.g. 20 mL) containing 2 mL methanol. Importantly, during studies following the semi-static test setup water samples were taken from both, the aged and fresh medium (prior to and after media exchange, respectively). After vigorously mixing, the medium samples were transferred to the analytical laboratory for further sample preparation and analysis on the same day or were immediately stored at ≤ -18°C. For hexachlorobenzene (flow-through setup) water samples (2 x 50 mL) were siphoned from the test vessels (for example using a glass beaker) from a central point in the test chamber and 50 g (weighing on an appropriate balance) poured into a 60 mL glass vial (with a screw cap). The two samples

were instantly transferred to the analytical laboratory for further processing and analysis on the same day or immediately frozen at ≤ -18°C.

Sample analysis

Water and tissue samples containing Terbutry and Prochloraz were analyzed using LC-MS/MS for quantification. Hexachlorbenzene was analysed using GC-MS (in SIM mode). Water samples containing ^{14}C radiolabelled Prochloraz were analyzed for [^{14}C] content by liquid scintillation counting (LSC). Details pertaining to methods, and instrumentation, are provided in the HYBIT protocol (Annex 9).

Lipid determination

For determination of the lipid content of the test organisms, the lipid extraction method of Smedes (1999) adapted by Schlechtriem et al. (2012) was used. Small glass vials (7 mL) were stored over night at 75 °C in a drying cabinet, placed in a desiccator for additional 30 min and weighted (empty). They were used to pool the lipid extract. The amphipods were transferred into glass test tubes (at least 10 mL). After 200 µl of solution 1 (Cyclohexane / Isopropanol 5:4 (v/v)) were added to the tube, and the amphipods were homogenized for about 1 min with a homogenizer with Teflon pestle. The pestle was rinsed with 4.3 ml Solution 1, which were also collected in the tube. After that 2.75 ml of distilled water were added, the tube was vortexed and centrifuged (12 min, 1650 rpm). The organic phase was transferred into the small glass vial using a Pasteur pipette. After that 2.5 ml of solution 2 (Cyclohexane / Isopropanol 87:13 (v/v)) were added to the remaining aqueous phase, the tube was vortexed again and centrifuged under the same conditions. The organic phase was pooled with the first one and evaporated with nitrogen until only the lipid phase was left. The extract in the glass vial was 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 was determined with a microbalance (accuracy 0.001 mg) for a total lipid content by weight.

Lipid determination (pre-test)

Eight of the eleven ring test participants joined a preliminary test to validate the performance of the lipid content determination. In case of the remaining three labs, the appropriate lab equipment was not available to carry out the analysis. Hyalella samples were provided by Fraunhofer IME having a known lipid content (benchmark). Samples were analysed by all labs using the extraction protocol described before.

Calculations

The bioconcentration factor (BCF) was determined based on the measured test item concentrations in water samples collected during the uptake phase and *H. azteca* collected during the uptake phase as well as during the depuration phase of the study. The methods used for BCF determination were largely based on the methods described for fish in Annex 5 of the OECD test guideline 305 (OECD 2012a). In contrast to the BCF determination in fish, growth can be neglected in *H. azteca* BCF calculation due to the use of adult amphipods as shown in this study.

Determination of tissue concentration at steady state

The *H. azteca* tissue concentration at the very end of the uptake phase was compared to the determined concentrations at the sampling times before. A steady state tissue concentration was calculated as a mean concentration of those individual values that are in a ± 20% range.

TWA calculations (semi-static and flow-through exposure)

The calculation method of the time-weighted average water concentration was chosen depending on the exposure method. For semi-static exposure experiments, the calculation method described in Annex 6 of the OECD TG 211 was applied (OECD 2012b). For the evaluation of flow-through exposure experiments, concentrations were multiplied with a weighing factor that represents the time span that this concentration was measured at. Finally,

the sum of all weighed concentrations was divided by the sum of the total exposure duration (Schlechtriem et al. 2019).

BCF_{ss} calculation

Steady state BCFs were calculated as the quotient of the *H. azteca* tissue concentration at steady state and the TWA of the test medium applied during the uptake phase.

BCF_k calculation – simultaneous (via bcmfR) and sequential determination

The kinetic BCF was calculated using the bcmfR package for R in the version 04.18 provided by Tom Aldenberg. This package was proposed as standardized method to evaluate bioconcentration studies in the Guidance Document for the OECD TG 305. In past studies, it was shown that this tool can also be used for bioconcentration studies with *H. azteca* (Kosfeld et al. 2020) and accordingly it was decided to use the tool for a standardized evaluation of the ring test results. The underlying model the concentration data was fitted to is the following:

$$C_{H.azteca}\,(t) = TWA * \frac{k_1}{k_2}\,(1 - e^{(-k_2 * t)}) \qquad \text{[Equation 1]}$$

In some cases the R calculation can fail, for example in cases when too many tissue concentration data points were below the analytical detection limit and cannot be considered for the calculation. In such cases, a manual calculation approach was performed. In contrast to the bcmfR package, which utilizes a sequential determination of the uptake and depuration constants k_1 and k_2, the manual approach is a sequential one that first calculates the depuration rate k_2 from the depuration data and then calculates the uptake rate from the uptake data, TWA and k_2 value. The depuration data was then fitted to a first order exponential decay model:

$$C_{H.azteca}\,(t) = a * e^{(-k_2 * t)} \qquad \text{[Equation 2]}$$

To derive the uptake rate k_1, the previously obtained k_2 value was inserted into equation 1 and the uptake concentration data was fitted to the equation.

Matlab 2018b was used to fit the data based on the sequential method.

In the past studies it was seen that the BCF_k values determined with the above described sequential method are well comparable with the BCF_k values determined in the untransformed fit with the bcmfR modeling approach. Accordingly, the untransformed fit of the bcmfR evaluations was used for all comparisons.

Lipid normalization

Both bioconcentration factors (BCF_{SS} and BCF_k) were lipid normalized to a tissue lipid content of 5%. For this the BCF was divided by the determined total lipid content and then multiplied with 5.

Error propagation

All BCF values were furthermore evaluated for their uncertainties. For this, the general law of error propagation without consideration of covariance was applied as described in Schlechtriem et al. 2019. Differences to the mentioned paper lie in the source of the errors for the k_1 and k_2 values which were derived from the bcmfR fitting results, or from the Matlab fit in case of a sequential fit.

Validity criteria

For a test during the ring-trial to be valid the following conditions were applied:

- The water temperature variation is less than ± 2°C, because large deviations can affect biological parameters relevant for uptake and depuration
- The concentration of dissolved oxygen does not fall below 60% saturation;
- The concentration of the test substance in the chambers is maintained within ± 20% of the mean of the measured values during the uptake phase;
- The concentration of the test substance is below its limit of solubility in water, taking into account the effect that the test water / medium may have on effective solubility;
- The mortality or other adverse effects/disease in treated *H. azteca* is less than 20% at the end of the test.

RESULTS

Husbandry establishment

Prior to the ring test experiments, a husbandry for *H. azteca* was established to ensure sufficient supply of animals throughout the test period in four labs. In six labs, an established husbandry was already present; one lab was supplied with "on-demand" *H. azteca* from the Fraunhofer IME facility.

The HYBIT protocol provided all details to establish the *H. azteca* husbandry including details on the proper selection of husbandry vessels, aeration, and feeding. All four labs were able to establish a husbandry that was able to produce enough offspring for bioconcentration tests. Two labs received additional *H. azteca* for their test starts. One lab had to postpone their husbandry establishment due to the COVID-19 pandemic and did not have enough amphipods for two tests in a short time frame. In another case, the lab had to repeat some experiments and needed additional supply with amphipods.

Lab participation and experimental setups

Eleven different labs participated in the main part of the experimental phase. Most labs conducted two different bioconcentration experiments, individual labs performed a single one, and other labs contributed three different experiments (Table 2). In total, 24 different experiments were provided for three different substances, whereas one substance (Prochloraz) was also tested as radiolabeled compound in two of these 24 experiments. Two different test set-ups were used. The potential effects of the different methods could be evaluated in case of the substance Prochloraz, which was tested regularly with both, the semi-static and the flow-through application.

Experimental conditions

Experimental conditions were monitored throughout the studies. The results of the measurements (temperature, pH, dissolved oxygen) presented in Annex 5 confirmed the

suitability of the test protocol to maintain acceptable conditions for the amphipods during the BCF studies. In several studies the concentration of dissolved oxygen dropped temporarily below 60%. However, this had obviously no effect on the condition of the invertebrates and a reduction of the minimum acceptable concentration of dissolved oxygen (validity criterion) from 60 to 50% should be thus considered. For individual studies, total organic carbon contents were recorded in the test vessels but never exceeded the threshold value of 10 mg/L. Mortality was generally below 20% in the different studies which was compensated by the addition of extra amphipods (additional 20%) at the start of the test. Only in three cases the number of amphipods was not sufficient for complete sampling.

Weight and lipid content of *H. azteca*

The average weight of the amphipods used for the bioconcentration tests and their lipid contents were determined. As shown in Figure 1, the size of the invertebrates was very different leading to large range of sample wet weight from around 20 to 100 mg. Data from all sampling events (start and end of uptake phase and end of depuration phase) were considered in the mean calculations. The lipid content of the amphipods was determined at three different times in all experiments (Annex 8). The mean lipid content of 2.2 ± 0.15% determined in the preliminary phase of the ring test served as orientation for the interpretation of the lipid data from the main tests. Lipid values were considered realistic and valid if the following aspects were met: 1) Mean lipid value < 5% 2) Relative SD < 30% 3) At least two replicates per sampling time could be evaluated and the lipid content determined.

In five out of the 24 experiments conducted no reliable or realistic lipid contents could be evaluated. Since these three labs received their *H. azteca* from the Fraunhofer IME lab, it was decided to assume a mean lipid value of 2.2 ± 0.5% equivalent to the benchmarking samples analysed during the lipid-determination pre-test. Mean lipid contents calculated throughout

the different experiments (all experiments) ranged from 1.4 ± 0.21% to 3.8 ± 0.60% as listed in Annex 8.

Water concentrations

Water concentrations were measured during the uptake phase of the bioconcentration experiments and ideally also at the onset of the depuration phase to ensure no substance was carried over. Time-weighted averages (TWA) for each exposure were calculated and furthermore, it was checked whether this TWA was in a ± 20 % range. Figure 3 summarizes the calculated TWAs for each experiment. If this range was crossed by any individual water concentration measurement, the respective concentration and the time when it was determined were listed (Annex 6). In only four out of 24 studies the ± 20% TWA concentration range was missed. In most cases, the ± 20% TWA concentration range was crossed at the start of the experiment at t = 0. The results show, that the HYBIT test system allows the application of constant water concentrations during the uptake phase.

***Hyalella azteca* tissue concentrations & steady state conditions**

Ideally, the uptake phase should be long enough to assure that the amphipods reach a steady state situation with their environmental conditions. In the OECD TG 305 for bioconcentration experiments with fish a steady state is defined as follows: "*A steady-state is reached in the plot of test substance in fish (C_f) against time when the curve becomes parallel to the time axis and three successive analyses of C_f made on samples taken at intervals of at least two days are within ± 20% of each other, and there are no significant increases among the three sampling periods.*" For the following evaluation, steady-state concentrations were calculated from the tissue concentration at the end of the uptake phase and the preceding tissue concentrations that fall within a ± 20% range of the tissue concentration at the end of the uptake phase (OECD 2012a). The amphipod concentrations at steady state are summarized in Figure 3. Furthermore, the duration to reach steady state conditions in the different studies are

presented in Annex 6 showing clear differences between some of the tests. BCF_{ss} calculated from the steady-state tissue concentrations and the corresponding TWAs are listed in Annex 6.

***Hyalella azteca* tissue concentration development over time**

The development of the tissue concentrations in the amphipods over time for the test compounds is presented in Figure 2. The plots underline the observations in regard to the steady-state durations described in Annex 6. The related uptake (k_1) and depuration (k_2) rates are presented in Figure 3.

Calculation of BCF_k

Annex 6 lists all parameters that were calculated for the different experiments using the bcmfR package for R. BCF_k values calculated from the uptake and depuration rates are presented in Figure 4 for the non-radiolabeled test compounds and range from 20-62 for Terbutryn, from 128 – 292 for Prochloraz, and 18544 to 32064 for HCB. Of the seven Terbutryn experiments that were conducted, only six could be evaluated via the bcmfR package for R. The long depuration experiment of lab 01 could not be evaluated by the R package, hence the kinetic parameters k_1 and k_2 (listed in Annex 6) were determined manually via the sequential approach in accordance with the description in Annex 5 of the OECD TG 305.

For Prochloraz, which was tested in 10 BCF studies under semi-static and flow-through conditions, no difference between the mean of the BCF values obtained during the six semi-static and the four flow-through studies could be detected (Figure 4).

In total, five different bioconcentration experiments with HCB were conducted. In one lab, a set of juvenile amphipods was additionally exposed to the test substance. The plots indicate that steady state was not reached in the HCB experiments. This impression is underlined by the comparison of the respective BCF_{SS} and BCF_k values (c.f. Annex 6). The BCF_{SS} values

451 are consistently lower than the corresponding BCF_k values, which according to the OECD TG
452 305 is an indication that steady-state has not been reached in the experiment.

453 The inter-laboratory variability (coefficient of variation) in the resulting BCF_{kL} was 34.4 %,
454 30.9 % and 28.1 % for all studies performed on Prochloraz, Terbutryn and HCB, respectively.

455 **Lipid-normalized BCF_k values**

456 As advised in Schlechtriem et al. 2019 a normalization of BCF values to 5% total lipid
457 content should be performed. However, the performance of the lipid determination itself may
458 need some practice which is why a preliminary lipid determination ring trial had been
459 initiated (c.f. Annex 7). While for five studies of the main ring trial later an empirically
460 determined lipid content value for the normalization procedure was required due to strongly
461 deviating fat contents, for the remaining 19 BCF experiments lipid values were available that
462 could be utilized. The lipid normalized BCF_k values are presented in Figure 4. Lipid
463 normalization was leading to increased BCF_k values. The uncertainties of Hyalella BCF
464 values were calculated by the general law of propagation.

465 **Comparison of radiolabel and non-radiolabel exposure with Prochloraz**

466 The plots in Figure 2 visualize the differences in the uptake of Prochloraz in the radiolabeled
467 experiments compared to the non-labeled ones. The plots for the radiolabel experiments at the
468 bottom of the figure indicate that no steady state was reached when the uptake of total
469 radioactivity, which also includes biotransformation products, was evaluated. However, in a
470 semi-static approach with radiolabeled Prochloraz in addition to the analysis for total
471 radioactivity in the amphipods' tissue, an additional analysis for the parent substance
472 prochloraz was performed. The determined BCF_k and BCF_{kL} values of the analysis for the
473 parent substance agree with the mean BCF values calculated from the data in the main test
474 (non-radiolabeled compound) which is graphically displayed in Figure 5.

Comparison BCF_k/BCF_{ss}

A comparison of BCF_{SS} and BCF_k values can give insight into the quality of the results according to paragraph 79 of the OECD TG 305. In all experiments with Prochloraz similar values were calculated for the BCF_{SS} and BCF_k values which indicates that steady-state was reached in the experiments despite differing times until steady-state were observed. The Terbutryn BCF values show a similar picture to the Prochloraz ones. An exception was a BCF value which was calculated based on an experiment with an extended depuration phase (119 hrs). For the respective dataset a fitting via the bcmfR package was not possible, accordingly a manual fit with the sequential method was performed. In this case, the resulting BCF_k is lower than the corresponding BCF_{SS}, which indicates a non-ideal fit. In all HCB experiments the calculated BCF_{ss} values are visibly lower than the corresponding BCF_k values. This underlines that steady state obviously has not been reached which is also supported by the respective concentration plots in Figure 2.

CONCLUSION & DISCUSSION

- The present ring trial builds on previous efforts (Schlechtriem et al. 2019; Kosfeld et al. 2020) to evaluate the reliability of the *Hyalella azteca* Bioconcentration Test (HYBIT) which allows to derive bioconcentration factors (BCF) using an invertebrate species.
- The present ring trial was accompanied by a large number of participating laboratories (11 labs) which could choose between two experimental set-ups. In addition to the common flow-through test design, a semi-static approach was applied. All laboratories were successful in establishing the test system with manageable effort. The HYBIT protocol was shown to be sufficiently robust to allow comparable performance even in laboratories that have no experience with either *H. azteca* or bioconcentration tests.
- Chemical analyses of the three test chemicals in water and *H. azteca* samples collected during the studies were mostly performed by the participating labs to allow a reliable assessment of user-associated sources of variability.
- Bioaccumulation is key to the regulation of chemicals in several jurisdictions. The ring test results demonstrate that comparable BCFs can be calculated from HYBIT experiment data which would have a consistent assessment impact e.g. under REACH.
- The lipid determination is a crucial part of the experiment. Therefore, care should be taken that the applied protocol delivers realistic values. The combined results of the preliminary lipid determination test and the main test show that a gravimetric lipid determination according to a downscaled Smedes (1999) protocol is feasible and replicable for *H. azteca* as part of bioconcentration tests with appropriate preparation. Alternative lipid determination protocols (e.g. colorimetric ones) may also be applicable, however the Smedes protocol is the one used for the OECD 305 studies in fish and may therefore deliver the most comparable data. Generally, the use of suitable equipment (scale with sufficient sensitivity), adequate training of the laboratory staff,

and the collection of a sufficient amount (mass) of *H. azteca* sampled per replicate are essential to obtain realistic results.

- Proper performance of bioconcentration studies requires knowledge of the toxicity of the test substances in order to derive appropriate test concentrations. Generally, information on the toxicity of chemicals to *H. azteca* are often missing. Therefore, an acute toxicity test design was developed which can help in the setting of suitable experimental conditions.
- For a test to be valid a set of criteria was defined including water temperature variation, concentration of dissolved oxygen, concentration of the test substance in the test chambers during the uptake phase, and the mortality of test animals during the study. The ring trial has shown that experimental conditions (temperature, dissolved oxygen) can be kept stable without much effort. In a few cases (four studies) concentration of the test substance in the chambers could not be maintained within ± 20% of the mean of the measured values (TWA) throughout the uptake phase. However, most of the concentrations that crossed the TWA range did so only for a single time and in a slight way and the results of the studies do not show any sort of divergence in comparison to the other studies. Mortality is another factor that should be monitored. Ideally, the mortality should not rise above a value of 20% in a bioconcentration test, as mortality indicates a poor health condition of the amphipods, which then again leads to unrepresentative physiological responses that alter the uptake and depuration kinetics. Mortality was generally below 20% in the different studies which was compensated by the addition of extra amphipods (additional 20%) at the start of the test. Only in three cases the number of amphipods was not sufficient for complete sampling.
- Overall, these study findings suggest that the *Hyalella azteca* Bioconcentration Test (HYBIT) is reliable. The test can be carried out with confidence to generate data

541 which may be used for the regulatory bioaccumulation assessment of chemicals.
542 During the ring trial only lipid accumulating chemicals were tested. Additional work
543 with chemical substrates representing a wider range of structures are required.
544 Bioconcentration studies with ionizable compounds and nanomaterials were carried
545 out as part of a UBA funded project (Schlechtriem et al. 2022, Kuehr et al. 2020).
546 • Hyalella BCF studies can be carried out with radiolabeled test compounds. However,
547 if radiolabelled substances are used, separation procedures such as thin-layer
548 chromatography (TLC) should be applied instead of TRR methods such as combustion
549 or LSC to allow calculation of BCF values which are directly comparable to a BCF
550 derived by specific chemical analysis of the parent substance (Raths et al. 2020).
551 • When a laboratory has no previous experience with the test or experimental conditions
552 have been changed the use of reference substances of known bioconcentration
553 potential such as the test chemicals applied in the ring trial would be useful in
554 checking the experimental procedure.
555 • In order that HYBIT as alternative test methods can be used systematically for
556 assessing the bioaccumulation potential of chemicals, an Integrated Test Strategy
557 (ITS) is required considering the opportunities and limitations of the new test system.

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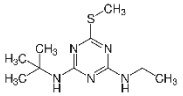
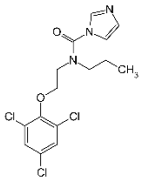
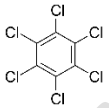
625
626

627 **TABLES**

628 Table 1**:** Details of the test substances used in the HYBIT ring test.

Substance name	CAS No.	Molecular formula	Molecular weight (g/mol)	Solubility in water	Log K_{OW}	*H. azteca* BCF in the literature (as BCF_{KL})
Terbutryn	886-50-0		241.36	22 - 58 mg/L (at 20°C)[1]	3.48 - 3.74	76 – 78 [Kosfeld et al. 2020]
Prochloraz	67747-09-5		376.67	23.6 - 42.9 mg/L (at pH 6 - 9 & 20 - 25°C)[2]	3.53 - 4.39 (at pH 6 - 9 & 20 - 25°C)	299 – 308 [Kosfeld et al. 2020]
HCB	118-74-1		284.76	5 - 6 µg/L (at 25 - 26 °C)[3]	5.37 - 5.73	25,704 [Schlechtriem et al. 2019]

629

[1] Terbutryn EQS dossier (2011)
[2] Registration Report Mirage 45 EC (BVL, 2011)
[3] EQS Substance Data Sheet for Hexachlorobenzene

630 Table 2**:** Overview over participating labs and their conducted bioconcentration experiments for the ring test, respectively.

Lab number	Test 1		Test 2		Test 3	
	Substance	Exposure	Substance	Exposure	Substance	Exposure
01	Prochloraz	semi-static	Terbutryn	semi-static (long uptake phase)	Terbutryn	semi-static (long depuration phase)
02	Prochloraz	semi-static	Terbutryn	semi-static		
03	Prochloraz	semi-static	Terbutryn	semi-static		
04	Prochloraz	semi-static	Terbutryn	semi-static		
05	Prochloraz	semi-static	Terbutryn	semi-static		
06	Prochloraz	semi-static	Terbutryn	semi-static	Prochloraz (^{14}C)	semi-static
07	Prochloraz (^{14}C)	Flow-through				
08	Prochloraz	Flow-through	HCB	Flow-through		
09	Prochloraz	Flow-through	HCB	Flow-through		
10	Prochloraz	Flow-through	HCB	Flow-through		
11	Prochloraz	Flow-through	HCB (adult H.a.)	Flow-through	HCB (juvenile H.a.)	Flow-through

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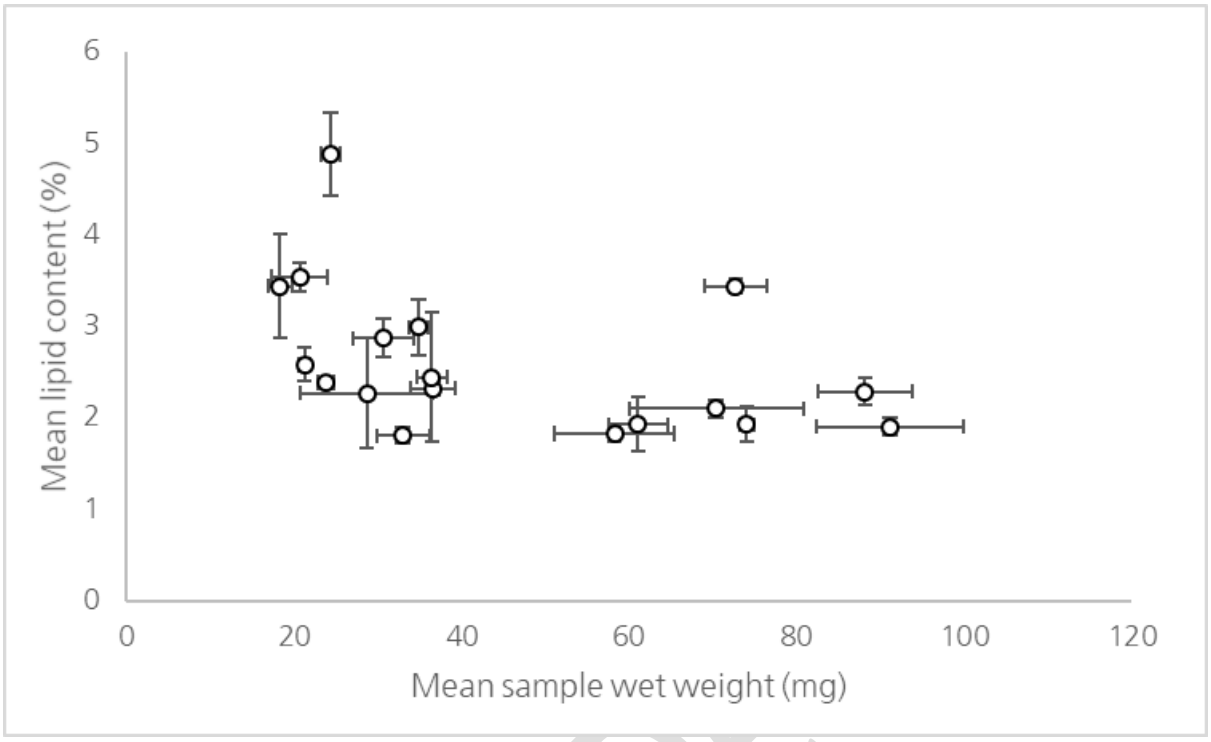
632

633 **FIGURES**



634

635 **Figure 1:** Average weight of amphipods used for the bioconcentration tests and their lipid
636 contents. Comparison of mean tissue wet weight and determined mean lipid content. Error
637 bars show the standard deviation (n=3). Note that with decreasing sample mass (< 50
638 mg/sample) increased standard deviations for lipid measurements were observed in several
639 cases.

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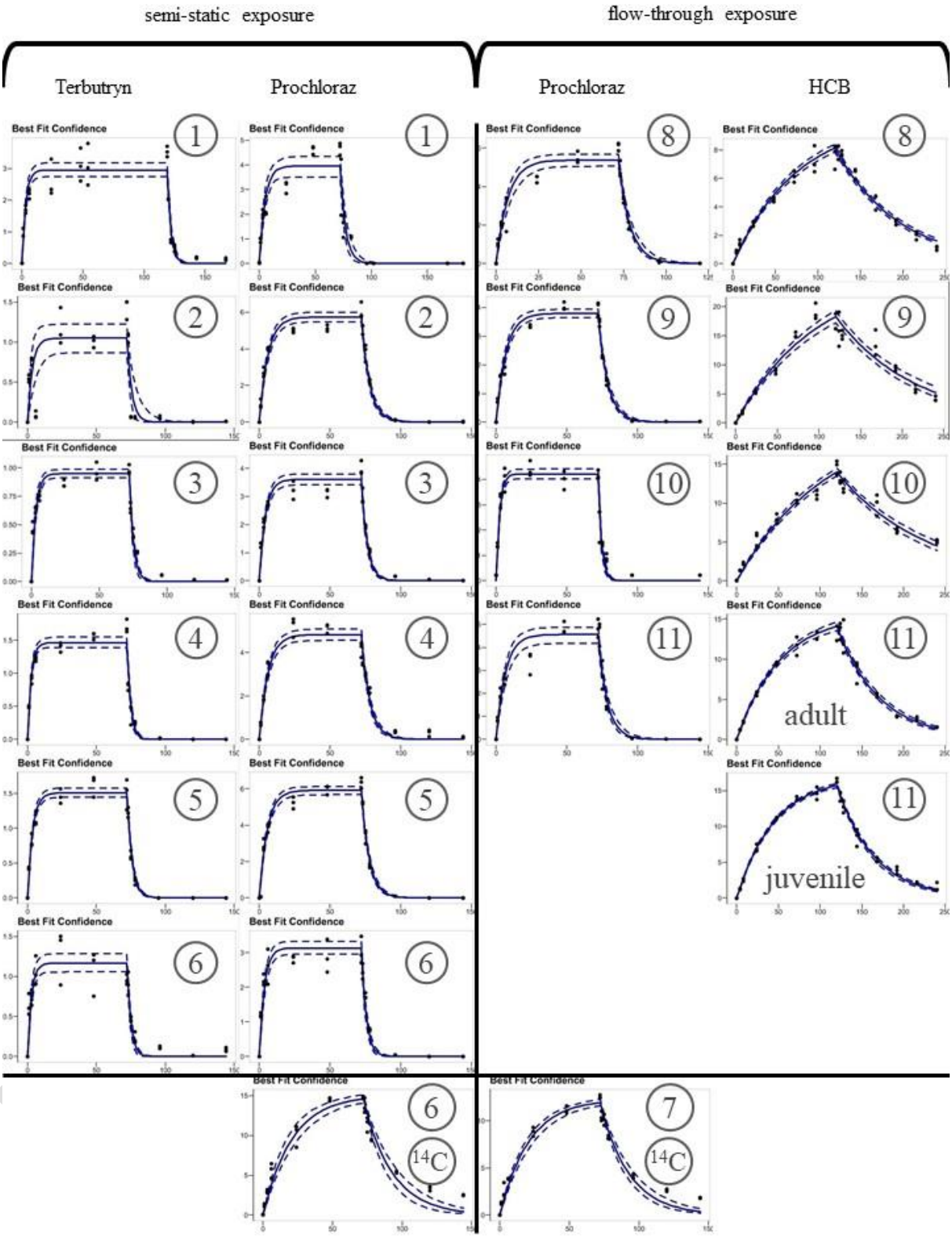

642

643 **Figure 2:** Concentration profiles over time for all experiments conducted in the HYBIT ring
644 test. Numbers in circles displayed next to each plot refer to the lab that conducted the study.
645 The x-axis displays the time in hours, the y-axis the concentration of the respective test
646 substance in *H. azteca* in mg/kg.

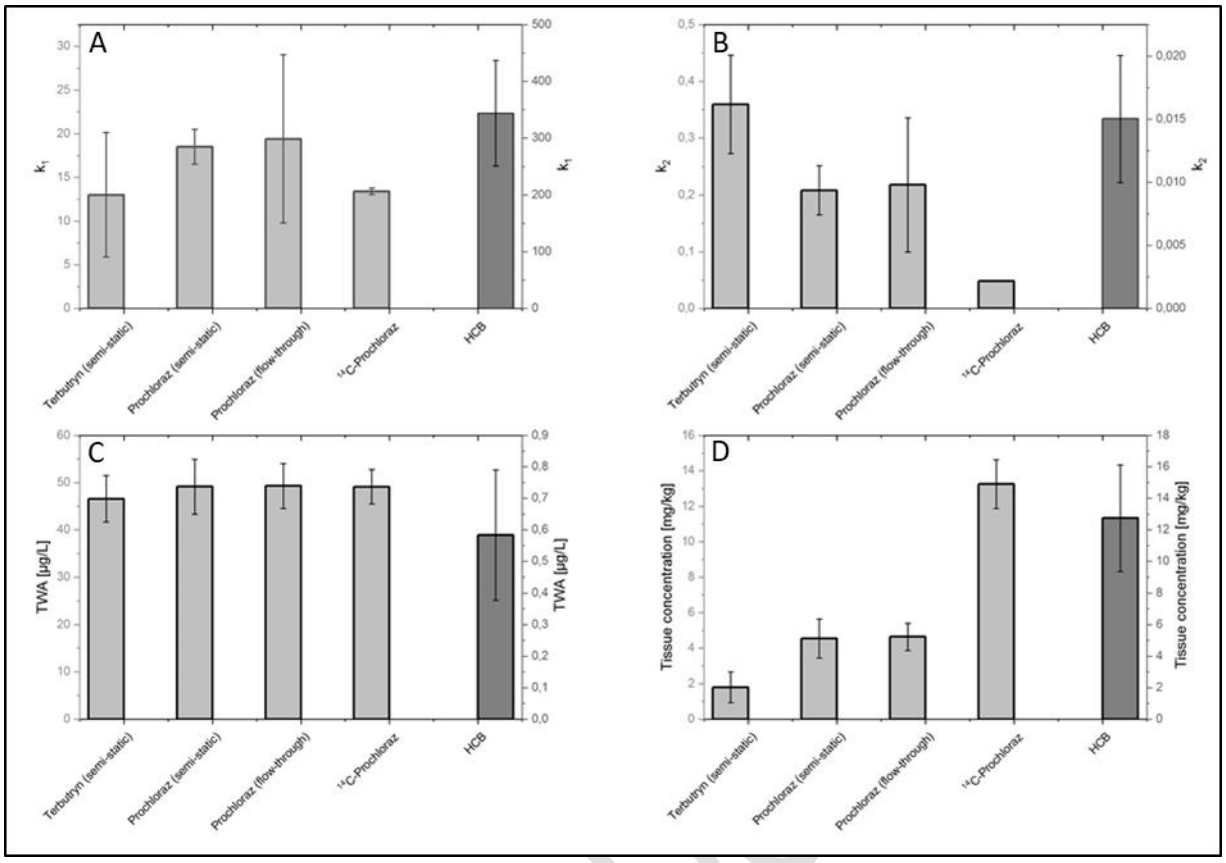

Figure 3: Results of the bioconcentrations experiments conducted in the HYBIT ring test. [left Y-axes: Terbutryn semi-static (n=7), Prochloraz semi-static (n=6), Prochloraz flow-through (n=4); ^{14}C Prochloraz (n=2); right Y-axes: HCB flow-through (n=5)]. Uptake rates k_1 (A), depuration rates k_2 (B), TWA, time-weighted average water concentrations (C) and tissue concentrations (D) measured under steady-state conditions are presented with standard deviations, respectively.

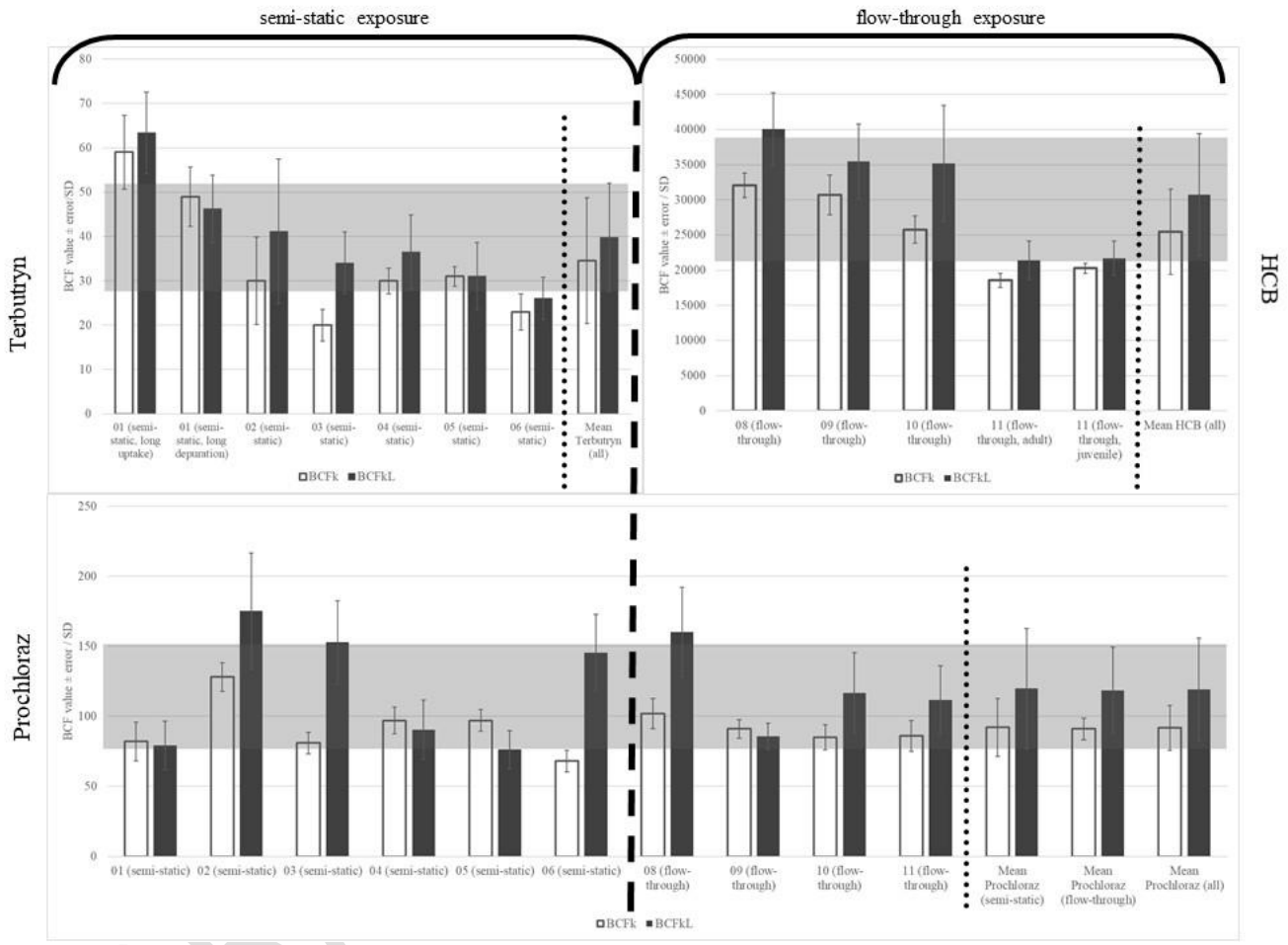

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Figure 4: Comparison plots for all BCF_K (white bars) and BCF_{KL} (black bars) values determined in the HYBIT ring test. Grey bars display the standard deviation of the mean of all BCF_{KL} values, the error bars of individual BCF_K and BCF_{KL} values display the BCF error calculated by the general law of propagation. Mean BCF values with standard deviation are presented.

ANNEX 1: ABBREVIATIONS

^{14}C – Carbon-14 radiolabeled

bcmfR – Name of the R toolkit for BCF calculation

BCF – Bioconcentration factor

BCF_K – Kinetic bioconcentration factor

BCF_{KL} - Kinetic bioconcentration factor, lipid normalized

BCF_{SS} – Steady-state bioconcentration factor

BCF_{SSL} – Steady-state bioconcentration factor, lipid normalized

C_{H-} Concentration measured in Hyalella

C_{W-} Concentration measured in water (test medium)

HCB – Hexachlorobenzene

HYBIT – *Hyalella azteca* bioconcentration test

IME – Institute for Molecular Biology and Applied Ecology

k_1 – Uptake rate

k_2 – Depuration rate

Lab – Laboratory

REACH - Registration, Evaluation, Authorisation and Restriction of Chemicals

TG – Test guideline

TRR - Total radioactive residue

TWA – Time weighted average

ANNEX 2: PARTICIPATING LABORATORIES (IN FREE ORDER)

- L´Oréal, France
- Eurofins, Germany
- BT, Italy
- INERIS, France
- IES, Switzerland
- BASF, Germany
- Noack, Germany
- IBACON, Germany
- FhG-IME, Germany
- UBA, Germany
- ECT, Germany

ANNEX 3: TOXICITY TEST WITH HYALELLA AZTECA AS PRELIMINARY EVALUATION FOR BIOCONCENTRATION TESTS

Since toxic effects are not desired and should be avoided in bioconcentration studies (OECD, 2012), 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 substance toward aquatic invertebrates is not always available. 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 substance characteristics do not allow for a semi-static exposure, the test setup may have to be changed to a flow-through one.

The protocol is based on an evaluation that has been performed by three different laboratories for the substance prochloraz (CAS: 67747-09-5). All ring test partners have used this substance in bioconcentration studies in the ring test, accordingly a suitable exposure concentration was of high priority.

Material:

- Glass aquarium (as water bath)
- Beaker (250 mL)
- Water heating element
- Shortened stainless-steel mesh shelters
- DECOTABs
- Artemia sieves
- Adult *H. azteca* (> 2 months old)

718 Test setup:

719 • 1 control
720 • 5 concentrations (treatments)
721 • 6 replicates per control/treatment
722 • 20 *H. azteca* per replicate
723 • Exposure duration: Approx. the planned duration of the uptake phase in the
724 bioconcentration test (here: 4 days / 96 hrs)
725 • Exposure method: semi-static (change to flow-through, if necessary)
726 • Daily medium renewal, temperature & O_2 saturation & pH determination
727 • Randomized placement of beakers in water bath
728 • Daily feeding with DECOTABs, ¼ cube per day per beaker
729 • Daily determination of water concentration (fresh and aged medium)
730 • Daily count of alive and, if visible, dead *H. azteca* in each beaker

731 **Table 1:** Concentrations applied in the toxicity range-finder test for Prochloraz with *H.*
732 *azteca*. Selection based on *D. magna* EC 50 (48 hrs) of 9.23 mg/L [Salesa et al. 2022] and the
733 exposure concentration of 50 µg/L in the *H. azteca* bioconcentration tests in Kosfeld et al.
734 2020. A spacing of approx. 3.5 is used between all concentrations.

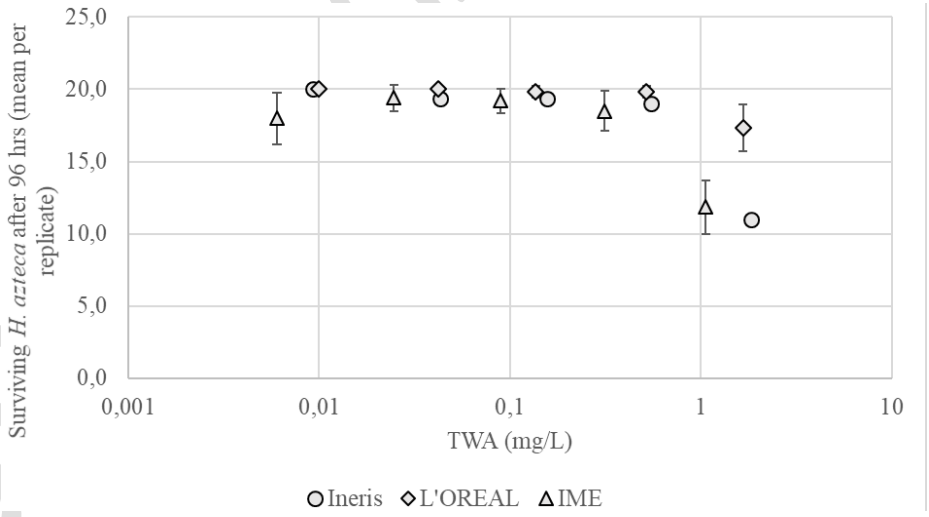
Scenario	**Prochloraz in medium (mg/L)**
Concentration 1	2.143
Concentration 2	0.612
Concentration 3	0.175
Concentration 4	0.050
Concentration 5	0.014
Control	0.000

735 Pooling option:

736 With 36 beakers in the test, daily media renewal and a determination of fresh and aged media
737 concentrations of the test substance, a considerable number of samples will be generated.
738 'Sample pooling' can help to reduce the number of samples for analyses. Aliquots (5mL)
739 collected from each beaker (total of 30 mL) should be sufficient to determine the average
740 parameters of each treatment. This option should only be selected, if there are no indications
741 that the treatment differs significantly from each other.

742 Results from the prochloraz experiment:

743 The preliminary toxicity test with prochloraz has validated that the exposure concentration of
744 0.05 mg/L that was used in Kosfeld et al. 2020 is safe and can be used in the ring test. First
745 toxic effects could be seen only in concentrations of > 1 mg/L Prochloraz in the medium after
746 an exposure time of 96hrs.



747

748 **Figure 1:** Results from the prochloraz toxicity test with *H. azteca*. Displayed are the average
749 numbers of surviving *H. azteca* per beaker in each of the applied concentrations. Prochloraz
750 concentration in the medium are shown as time-weighted average (TWA) results on the x-
751 axis. Error bars display the standard deviation, based on 5-6 replicates per concentration.

752 **Table 2:** Mortality rates determined in the preliminary toxicity tests of the substance

753 prochloraz.

Lab	Test concentration, nominal (mg/L)	Test concentration, TWA (mg/L)	Mortality, mean (%)
Ineris	Control	0	0.8
	0.014	0.009	0.0
	0.05	0.043	4.9
	0.175	0.156	4.1
	0.612	0.551	5.8
	2.143	1.84	46.8
L'OREAL	Control	0	1.7
	0.014	0.010	0.0
	0.05	0.042	0.0
	0.175	0.136	0.8
	0.612	0.519	0.8
	2.143	1.660	13.3
IME	Control	0	3.3
	0.014	0.006	10.0
	0.05	0.025	3.0
	0.175	0.090	4.0
	0.612	0.314	7.5
	2.143	1.060	40.8

754

755

Validity criteria

Based on the results of the toxicity test with prochloraz. the following validity criteria for a preliminary. acute toxicity test with *H. azteca* are proposed.

- Control mortality < 10%

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.

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Kosfeld V. Fu Q. Ebersbach I. et al (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 39:1813–1825. https://doi.org/10.1002/etc.4791

OECD (2012) Test No. 305: Bioaccumulation in Fish: Aqueous and Dietary Exposure. OECD Guidelines for the Testing of Chemicals. Paris. France.

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ANNEX 4: SAMPLING SCHEDULE

Sampling schedule: Terbutryn – semi-static test setup

	Hours	*H. azteca* samples. tissue analysis	*H. azteca* samples. lipid	Test medium	Test medium
Uptake phase	0	3 x 20 H.a.**	3 x 10 H.a.**	2 x 10	
	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	2 x 10
	48	3 x 20 H.a.		2 x 10	2 x 10
	72	3 x 20 H.a.	3 x 10 H.a.		2 x 10
Depuration phase	1 (73)	3 x 20 H.a.		2 x 10	
	3 (75)	3 x 20 H.a.			
	6 (78)	3 x 20 H.a.			
	24 (96)	3 x 20 H.a.		2 x 10	2 x 10
	48 (120)	3 x 20 H.a.		2 x 10	2 x 10
	72 (144)	3 x 20 H.a.	3 x 10 H.a.		2 x 10
		3 x 20 = 60** 12 x 3 x 20 = **720 H.a.**	1 x 3 x 10 = 30** 2 x 3 x 10 = **60**	12 x 10 mL	12 x 10 mL

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

** Hyalella collected from the batch of male amphipods just before test animals were placed in the test chamber!

***further animals (approx. 20%) were added to compensate potential losses.

Sampling schedule: Prochloraz – semi-static test setup

	Hours	***H. azteca* samples. tissue analysis**	***H. azteca* samples. lipid**	**Test medium**	**Test medium**
Uptake phase	0	3 x 20 H.a.**	3 x 10 H.a.**	2 x 10	
	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	2 x 10
	48	3 x 20 H.a.		2 x 10	2 x 10
	72	3 x 20 H.a.	3 x 10 H.a.		2 x 10
Depuration phase	1 (73)	3 x 20 H.a.		2 x 10	
	3 (75)	3 x 20 H.a.			
	6 (78)	3 x 20 H.a.			
	24 (96)	3 x 20 H.a.		2 x 10	2 x 10
	48 (120)	3 x 20 H.a.		2 x 10	2 x 10
	72 (144)	3 x 20 H.a.	3 x 10 H.a.		2 x 10
		3 x 20 = 60** 12 x 3 x 20 = **720 H.a.**	1 x 3 x 10 = 30** 2 x 3 x 10 =	12 x 10 mL	12 x 10 mL

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

** Hyalella collected from the batch of male amphipods just before test animals were placed in the test chamber!

***further animals (approx. 20%) were added to compensate potential losses.

Sampling schedule: Prochloraz – flow-through test setup

	Hours	***H. azteca* samples. tissue analysis**	***H. azteca* samples.**	**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.		
	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
		3 x 20 = 60** 12 x 3 x 20 = **720 H.a.**	1 x 3 x 10 = 30** 2 x 3 x 10 =	14 x 10 mL

* Water concentration in the test medium was analysed daily.

** Hyalella collected from the batch of male amphipods just before test animals were placed in the test chamber!

***further animals (approx. 20%) were added to compensate potential losses.

Sampling schedule: HCB – flow-through setup

	Hours	*H. azteca* samples.	*H. azteca*	Test medium
Uptake phase	0	3 x 20 H.a.*	3 x 10 H.a. *	2 x 50 mL
	4	3 x 20 H.a.		
	8	3 x 20 H.a.		
	24 (day 1)	3 x 20 H.a.		2 x 50 mL
	48 (day 2)	3 x 20 H.a.		2 x 50 mL
	72 (day 3)	3 x 20 H.a.		2 x 50 mL
	96 (day 4)	3 x 20 H.a.		2 x 50 mL
	120 (day 5)	3 x 20 H.a.	3 x 10 H.a.	2 x 50 mL
Depuration phase	4 (124)	3 x 20 H.a.		2 x 50 mL***
	8 (128)	3 x 20 H.a.		
	24 (144)	3 x 20 H.a.		
	48 (168)	3 x 20 H.a.		
	72 (192)	3 x 20 H.a.		
	96 (216)	3 x 20 H.a.		
	120 (240)	3 x 20 H.a.	3 x 10 H.a.	
		1 x 3 x 20 = 60 14 x 3 x 20 = **840**	1 x 3 x 10 = 30 2 x 3 x10 = **60**	14 x 50 mL

* Hyalella collected from the batch of male amphipods just before test animals were placed in the test chamber!

** further animals (approx. 20%) were added to compensate potential losses.

*** Additional water sampling during the depuration phase may have been required if the measured test substance concentration was > LOQ at the beginning of the depuration phase.

ANNEX 5: EXPERIMENTAL CONDITIONS

Table 1: Exposure media and experimental conditions during semi-static and flow through bioconcentration experiments.

Substance	Exposure	Lab No.	Exposure medium	Aeration	Temperature °C		pH		Oxygen saturation mg/L		Oxygen saturation %		*H. azteca* sample weight (mg) per 20 amphipods	
					Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
Terbutryn	semi-static#	01	Borgmann medium	no	24.3	25.0	8.1*	8.1*	8.64**	8.79**	95*	97.7*	45.89	69.07
	semi-static##	01	Borgmann medium	no	24.3	24.9	8.05*	8.1*	8.73**	8.82**	97*	98*	46.09	77.98
	semi-static	02	Borgmann medium	no	25.1	27.5	6.9	7.4	n.a.	n.a.	n.a.	n.a.	14.76	67.79
	semi-static	03	Borgmann medium	yes	23.5	24	7.85	8.2	6.21	8.72	62.1	87.2	59.16	85.62
	semi-static	04	Borgmann medium	no	23	25	7.5	8	6,68**	8,46**	82	100	25.02	74.29
	semi-static	05	Reconstituted medium	no	23.4	25.6	7.2	8.1	2.9	8.6	34.7	101.4	37.81	64.02
	semi-static	06	Elendt M4 medium + NaBr	yes	23	24.3	7.8	8.06	5.94	8.97	71	103	26.2	65.45
Prochloraz	semi-static	01	Borgmann medium	no	25.2	25.4	8.05*	8.15*	8.15**	8.9**	92.3*	98*	66.7	84.58
	semi-static	02	Borgmann medium	no	26.7	29	6.7	6.9	3.35	8,9*	41.9	111.3	39.67	134.5
	semi-static	03	Borgmann medium	yes	24.1	25.2	7.42	7.89	7.22	8.55	72.2	85.5	62.79	84.6

	semi-static	04	Borgmann medium	no	23	25	7.49	7.9	7,09**	8,46**	87	100	35.85	51.88
	semi-static	05	Reconstituted medium	no	24	26.4	7.3	8.2	4	8.5	49.6	102.9	30.12	48.46
	semi-static	06	Elendt M4 medium + NaBr	yes	22.6	25	7.61	8.05	6.81	8.46	83	102	53.78	73.18
	flow-through	08	Borgmann medium	yes	23	24	7.72	8.16	8.1	8.5	96,2**	99,1**	55.75	105.2
	flow-through	09	Borgmann medium	yes	22.9	23.5	7.16	7.76	7,46*	8,38*	89	99	66	102
	flow-through	10	ISO medium	no	28.5	65.28	7.65	8	8.83	9.65	103	112	23.5	25.4
	flow-through	11	Aerated, de-chlorinated tap water	yes	24.4	24.8	7.47	7.63	7.65	8.85	93.4	106.9	56.5	88
^{14}C-Prochloraz	semi-static	06	Elendt M4 medium + NaBr	yes	23	24	7.64	8.13	6.84	8.27	82	101	55.41	78.89
	flow-through	07	Borgmann medium	yes	23.6	24.7	7.6	8.1	8	10.1	96	121	38.9	64.5
HCB	flow-through	08	Borgmann medium	yes	24	26	8.03	8.31	7.17	8.1	86,8**	98**	59.22	91.43
	flow-through	09	Borgmann medium	yes	23.2	23.9	7.38	7.89	7,7**	8,2**	92	99	91.3	121.73
	flow-through	10	ISO medium	no	23.7	26.7	7.4	8	5.8	9.1	69	106	51.06	82.19
	flow-through	11	Aerated, de-chlorinated tap water	yes	25.1	25.5	7.22	7.66	8.15	9.3	101.2	111.9	56.82	83.15
	flow-through	11	Aerated, de-chlorinated tap water	yes	25	25.5	7.2	7.75	8.22	9.49	102.1	113.5	31.69	48.35

* determined in the fresh medium prior to the substance addition

** calculated values (Table used for calculations: https://pubs.usgs.gov/tm/09/a6.2/tm9a6.2.pdf)

long uptake phase (119h), regular depuration phase

regular uptake phase, long depuration phase (119h)

Citation for dissolved oxygen calculation table: U.S. Geological Survey, 2020, Dissolved oxygen: U.S. Geological Survey Techniques and Methods, book 9, chap. A6.2, 33 p., https://doi.org/10.3133/tm9A6.2. [Supersedes USGS Techniques of Water-Resources Investigations, book 9, chap. A6.2, version 3.0.]

ANNEX 6: RESULTS OF THE BIOCONCENTRATION EXPERIMENTS CONDUCTED IN THE HYBIT RING TEST.

Table 1: Time weighted average water concentrations, tissue concentrations at steady-state and steady-state duration.

Laboratory (exposure method)	Test substance	TWA (µg/L)	± 20 % TWA range (µg/L)	All concentrations in ± 20 % TWA range?	If ± 20 % TWA range was crossed. at which concentration?	Tissue concentration at steady-state (mg/kg)	Steady state duration
01 (semi-static)	Terbutryn[#]	49.90	39.92 – 59.88	Yes	-	3.086	24 – 119 hrs
01 (semi-static)	Terbutryn[##]	48.30	38.64 – 57.96	Yes	-	3.173	48 – 72 hrs
02 (semi-static)	Terbutryn	34.76	27.81 – 41.71	Yes	-	1.202	24 – 72 hrs
03 (semi-static)	Terbutryn	46.51	37.21 – 55.81	Yes	-	0.934	24 – 72 hrs

04 (semi-static)	Terbutryn	48.23	38.58 – 57.87	Yes	-	1.560	24 – 72 hrs
05 (semi-static)	Terbutryn	48.67	38.95 – 58.42	Yes	-	1.526	24 – 72 hrs
06 (semi-static)	Terbutryn	49.68	39.74 – 59.62	Yes	-	1.093	6 – 72 hrs
01 (semi-static)	Prochloraz[##]	48.35	38.68 – 58.02	Yes	-	4.642	48 – 72 hrs
02 (semi-static)	Prochloraz	44.57	35.66 – 53.49	Yes	-	5.687	24 – 72 hrs
03 (semi-static)	Prochloraz	44.34	35.47 – 53.21	Yes	-	3.452	24 – 72 hrs
04 (semi-static)	Prochloraz	50.03	40.02 – 60.03	Yes	-	4.832	24 – 72 hrs

05 (semi-static)	Prochloraz	61.22	48.79 – 73.46	Yes	-	5.844	24 – 72 hrs
06 (semi-static)	Prochloraz	46.26	37.01 – 55.52	Yes	-	2.842	6 – 72 hrs
06 (semi-static)	^{14}C-Prochloraz	52.72	42.18 – 63.26	Yes	-	14.63	48 – 72 hrs
07 (flow-through)	^{14}C-Prochloraz	45.53	36.43 – 54.64	Yes	-	11.86	48 – 72 hrs
08 (flow-through)	Prochloraz	53.04	42.43 – 63.65	Yes	-	5.797	48 – 72 hrs
09 (flow-through)	Prochloraz	41.52	33.21 – 49.82	Yes	-	3.787	24 – 72 hrs
10 (flow-through)	Prochloraz	49.29	39.43 – 59.15	No	65.87 and 67.94 at 0 hrs.	4.146	6 – 72 hrs

11 (flow-through)	Prochloraz	53.26	42.61 – 63.91	Yes	-	4.822	48 – 72 hrs
08 (flow-through)	HCB	0.316	0.253 – 0.379	No	0.396 at 0 hrs and 0.252 at 24 hrs	6.993	72 – 120 hrs
09 (flow-through)	HCB	0.833	0.667 – 1.0	Yes	-	17.058	72 – 120 hrs
10 (flow-through)	HCB	0.826	0.661 – 0.991	No	0.645 at 0 hrs and 1.106 at 120 hrs	12.075	72 – 120 hrs
11 (flow-through)	HCB[$]	0.841	0.673 – 1.010	Yes	-	12.674	72 – 120 hrs
11 (flow-through)	HCB[$$]	0.831	0.665 – 0.997	Yes	1.066 at 120 hrs	14.916	72 – 120 hrs

[#] = long uptake phase (120 h). regular depuration phase

[##] = regular uptake phase. long depuration phase (120 h)

[$] = adult *H. azteca* (> 2 months); [$$] = juvenile *H. azteca* (approx. 1 month old)

Table 2: Uptake rates (k_1). depuration rates (k_2) and kinetic and steady-state BCF values calculated with the R tool 'bcmfR', if not stated differently, followed by lipid normalization to a lipid content of 3%. BCF errors were calculated via error propagation.

Laboratory (exposure method)	Test substance	k_1	(± std. error)	k_2	(± std. error)	BCF_{ss}	Error	BCF_{ssL}	Error	BCF_k	Error
01 (semi-static)	Terbutryn[#]	19.95	1.995	0.338	0.033	62	36.1	66	12.4	59	8.3
01 (semi-static)	Terbutryn[##]	27.30	1.930	0.553	0.064	66	10.1	62	11.1	49**	6.7**
02 (semi-static)	Terbutryn	8.68	2.029	0.287	0.066	35	7.3	47*	14.4*	30	9.9
03 (semi-static)	Terbutryn	6.62	0.5	0.325	0.023	20	1.9	33	6.7	20	2.1
04 (semi-static)	Terbutryn	11.69	0.8	0.385	0.026	32	3.2	39	8.9	30	2.9
05 (semi-static)	Terbutryn	8.43	0.4	0.272	0.013	31	3.7	31	8.2	31	2.2
06 (semi-static)	Terbutryn	8.36	1.0	0.356	0.044	22	5.7	24	6.5	23	4.1
01 (semi-static)	Prochloraz[##]	16.63	2.0	0.204	0.024	96	9.10	93	15.6	82	14.0
02 (semi-static)	Prochloraz	21.83	1.3	0.170	0.009	128	15.9	174*	44.4*	128	10.3
03 (semi-static)	Prochloraz	19.57	1.3	0.240	0.016	78	12.4	146	33.9	81	7.8
04 (semi-static)	Prochloraz	16.04	1.2	0.166	0.011	97	12.1	91	22.3	96	9.6
05 (semi-static)	Prochloraz	17.42	1.0	0.180	0.010	95	9.4	75	14.0	97	7.8
06 (semi-static)	Prochloraz	19.49	1.6	0.288	0.023	61	11.3	132	31.3	68	7.8
06 (semi-static)	^{14}C-Prochloraz	13.75	1.0	0.048	0.004	278	9.3	333	63.6	286	30.9
07 (flow-through)	^{14}C- Prochloraz	13.01	0.8	0.048	0.003	260	23.1	355*	85.1*	271	24.4
08 (flow-through)	Prochloraz	14.73	1.1	0.145	0.011	109	9.7	173	32.9	101	10.8
09 (flow-through)	Prochloraz	13.52	0.7	0.148	0.008	91	15.3	86	15.9	91	6.8
10 (flow-through)	Prochloraz	36.05	2.7	0.422	0.032	84	18.1	115	35.5	85	9.1
11 (flow-through)	Prochloraz	13.29	1.2	0.155	0.014	91	8.8	118	24.0	86	10.9
08 (flow-through)	HCB	433.82	14.4	0.014	0.001	22130	4318	27662	6289	32064	1778
09 (flow-through)	HCB	230.20	16.1	0.010	0.001	20473	2657	23622	4164	30729	2823
10 (flow-through)	HCB	242.62	9.3	0.009	0.001	14619	3782	19935*	6805*	25780	1917
11 (flow-through)	HCB[$]	360.68	13.0	0.019	0.001	15070	1495	17389	2646	18544	1014
11 (flow-through)	HCB[$$]	451.39	11.2	0.022	0.001	17946	2295	19228	3201	20260	741

[$] = adult *H. azteca* (> 2 months); [$$] = juvenile *H. azteca* (approx. 1 month old)

* A default lipid content of 2.2 ± 0.3% was used due to unrealistic or non-available lipid determination results

** Calculation via bcmfR failed. kinetic BCFs were calculated manually (sequential method)

ANNEX 7: PRELIMINARY LIPID CONTENT DETERMINATION: RESULTS

Of the eleven ring test participants. eight were able to participate in the preliminary test on lipid content determination. In case of the remaining three labs. the lab equipment was not sensitive enough for the analysis. Hyalella samples were provided by Fraunhofer IME having a lipid content of 2.1 ± 0.1 % (benchmark). A comparison of the results is shown in Figure 1. Seven labs met this benchmark. two labs displayed deviations and were contacted for troubleshooting. A mean lipid content of 2.2 ± 0.15 % was calculated from the data that met the benchmark value. The mean lipid value for the full dataset was 2.6 ± 0.98 % (Annex 8)

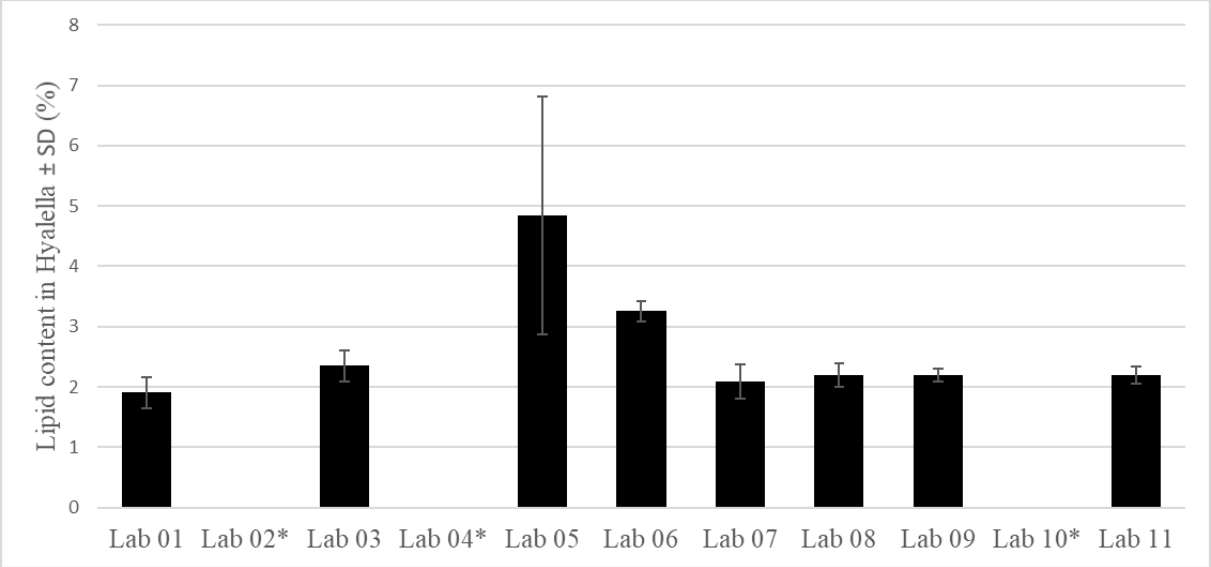

Figure 1: Results of the lipid determination preliminary test. Labs marked with an * did not participate in the preliminary test due to missing equipment.

ANNEX 8: LIPID CONTENT OF HYALELLA AZTECA DETERMINED IN EACH HYBIT BIOCONCENTRATION EXPERIMENT.

Lipid contents were determined gravimetrically by using the Smedes protocol. Results shaded in grey were considered unrealistic (e.g. negative lipid content values) pointing towards a problem with the weighing technique's sensitivity and were not used for further calculations.

Laboratory (exposure method)	Test substance	Mean lipid content at test start ± SD (%)	Mean lipid content at end of uptake phase ± SD (%)	Mean lipid content at end of depuration phase ± SD (%)	Mean lipid content over entire test duration ± SD and rel. SD (%)
01 (semi-static)	Prochloraz	3.0 ± 0.31	3.4 ± 0.49	2.8 ± 0.09	3.1 ± 0.43 (14.0%)
02 (semi-static)	Prochloraz	-0.25 ± 0.78	0.75 ± 0.67	-0.25 ± 1.37	-0.01 ± 1.13 (13112%)
03 (semi-static)	Prochloraz	1.9 ± 0.19	1.5 ± 0.14	1.4 ± 0.15	1.6 ± 0.27 (16.9%)
04 (semi-static)	Prochloraz	2.6 ± 0.18	3.0 ± 0.11	4.0 ± 0.58	3.2 ± 0.68 (21.3%)
05 (semi-static)	Prochloraz	3.4 ± 0.57	3.8 ± 0.65	4.2 ± 0.06	3.8 ± 0.60 (15.7%)
06 (semi-static)	Prochloraz	1.6 ± 0.03	1.2 ± 0.13	N/A	1.4 ± 0.21 (14.8%)
06 (semi-static)	^{14}C-Prochloraz	1.9 ± 0.30	2.7 ± 0.30	2.8 ± 0.14	2.5 ± 0.47 (18.9%)

07 (flow-through)	^{14}C-Prochloraz	4.9 ± 0.45	6.0 ± 1.52	4.3 ± 0.43	5.1 ± 1.19 (24.7%)
08 (flow-through)	Prochloraz	1.9 ± 0.10	1.7 ± 0.20	2.2 ± 0.38	1.9 ± 0.32 (16.5%)
09 (flow-through)	Prochloraz	3.4 ± 0.08	3.0 ± 0.06	3.1 ± 0.31	3.2 ± 0.26 (8.2%)
10 (flow-through)	Prochloraz	-0.1 ± 2.57	6.1 ± 5.69	4.1 ± 2.01	3.4 ± 4.56 (136%)
11 (flow-through)	Prochloraz	1.8 ± 0.09	2.6 ± 0.15	2.5 ± 0.30	2.3 ± 0.41 (17.6%)
01 (semi-static)	Terbutryn[#]	2.9 ± 0.21	2.6 ± 0.26	2.9 ± 0.10	2.8 ± 0.24 (8.4%)
01 (semi-static)	Terbutryn[##]	3.5 ± 0.16	3.0 ± 0.27	3.3 ± 0.01	3.2 ± 0.30 (9.3%)
02 (semi-static)	Terbutryn	-0.08 ± 0.91	-0.39 ± 1.07	0.5	-0.13 ± 0.97 (775%)
03 (semi-static)	Terbutryn	1.8 ± 0.08	1.5 ± 0.20	2.0 ± 0.34	1.8 ± 0.32 (18.5%)
04 (semi-static)	Terbutryn	2.3 ± 0.60	2.3 ± 0.34	2.9 ± 0.27	2.5 ± 0.52 (21.0%)
05 (semi-static)	Terbutryn	2.4 ± 0.70	3.2 ± 0.60	3.2 ± 0.44	3.0 ± 0.70 (23.4%)

06 (semi-static)	Terbutryn	2.9 ± 0.05	2.6 ± 0.23	1.0 ± 0.13[Π]	2.7 ± 0.23 (8.45%)
08 (flow-through)	HCB	2.1 ± 0.09	2.3 ± 0.20	2.7 ± 0.14	2.4 ± 0.28 (11.9%)
09 (flow-through)	HCB	2.3 ± 0.15	3.0 ± 0.10	2.7 ± 0.13	2.6 ± 0.31 (11.7%)
10 (flow-through)	HCB	6.8 ± 1.02	7.2 ± 0.74	9.0 ± 5.20	7.7 ± 3.24 (42.2%)
11 (flow-through)	HCB[$]	2.3 ± 0.08	2.8 ± 0.19	2.7 ± 0.34	2.6 ± 0.30 (11.6%)
11 (flow-through)	HCB[$$]	2.4 ± 0.05	2.9 ± 0.11	3.1 ± 0.12	2.8 ± 0.30 (10.8%)

[#] = long uptake phase (120 h). regular depuration phase

[##] = regular uptake phase. long depuration phase (120 h)

[$] = adult *H. azteca* (> 2 months)

[$$] = juvenile *H. azteca* (approx. 1 month old XYZ)

[Π] = Pale and sluggish *H. azteca.* excluded from mean calculation

Note: Lab 02 and 10 did not participate in the preliminary lipid content determination test (see Annex 7). In both labs the gravimetric determination of lipid contents resulted in unrealistic values during the main test which underlines the need for careful establishment of the methods.