Bioconcentration, Metabolism, and Spatial Distribution of $^{14}$C-Labeled Laurate in the Freshwater Amphipod *Hyalella azteca*

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Abstract: Regulatory assessment of the bioaccumulation from water is commonly based on bioconcentration factors (BCFs) derived from fish flow-through tests. Such experiments require many laboratory animals and are time-consuming and costly. An alternative test setup for organic, neutral compounds using the amphipod *Hyalella azteca* was recently suggested, resulting in BCF values which show a strong correlation with fish BCF data. In the present study, the bioconcentration potential of the ionic compound laurate was elucidated in *H. azteca*. The sodium salt of 1-$^{14}$C laurate was applied to *H. azteca* in a flow-through and a semistatic approach. Because of rapid biodegradation, a semistatic approach with frequent medium replacements was required to ensure a stable medium concentration. Laurate was also rapidly metabolized by *H. azteca*. A large proportion of the total radioactivity measured in the amphipod tissue was not extractable, suggesting that mineralized laurate was accumulated in the calcified exoskeleton of *H. azteca*. This was confirmed in a further study using carbonate [$^{14}$C]. A lipid-normalized (5.0%) *Hyalella* BCF of 8.9 was calculated for laurate, measured as free fatty acids. The results of the bioconcentration studies with *H. azteca* confirm the low bioaccumulation potential of the test item previously observed in fish. However, more organic ionic compounds with various properties need to be tested to assess whether a general correlation between fish and *Hyalella* BCF data exists. Environ Toxicol Chem 2020;39:310–322. © 2019 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals, Inc. on behalf of SETAC.

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INTRODUCTION

Bioaccumulation is one of the central processes in ecotoxicology and describes the enrichment of xenobiotics in organisms. The ultimate decisive bioaccumulation criterion as part of the regulatory persistent, bioaccumulative, and toxic (PBT) assessment is the bioconcentration factor (BCF). The BCF reflects the proportion of the internal concentration of a test organism under steady-state conditions compared to the concentration in the test medium (Registration, Evaluation, Authorisation and Restriction of Chemicals [REACH]; European Commission 2011). The determination of BCF values for regulatory use is commonly based on fish flow-through tests carried out according to guideline 305 (Organisation for Economic Cooperation and Development 2012b). Even though such experiments are well established, they usually require many (>100) laboratory animals, may last several weeks, and are costly. Ethics of animal testing are intensively discussed, especially in view of the 3-R principles (de Wolf et al. 2007; Russell and Burch 1959). Thus, alternative test methods would be of value.

An alternative test setup for organic, neutral compounds using the freshwater amphipod *Hyalella azteca* was recently suggested (Schlechtriem et al. 2019). *Hyalella azteca* is an abundant component of benthic communities in virtually all permanent freshwaters of North and Central America (Bousfield 1958; de March 1978) and is already well established as a test species in ecotoxicological testing (Environment Canada 1997; US Environmental Protection Agency 2000; Borgmann 2002). Because of the fast reproduction rate and growth of the amphipods, the culturing of *H. azteca* can easily provide the high numbers of animals required for bioconcentration studies. The
systematic investigation of the correlation between BCF values from fish and H. azteca showed a good correlation ($r^2 = 0.69$, $n = 148$, $p < 0.001$) between the 2 test systems for neutral organic compounds (Schlechtriem et al. 2019). However, there have been no studies so far elucidating the bioconcentration of ionic organic compounds in H. azteca.

The test item laurate is the anion of lauric acid (dodecanoic acid), a saturated medium-chain fatty acid with a chain length of 12 carbon atoms (C 12:0). The major industrial use of laurate is as the hydrophobic tail of various surfactants for hygienic and cosmetic applications (Tolls et al. 1994). Surfactants are usually rapidly degraded in wastewater-treatment plants. However, a certain fraction is still entering the environment (Ying 2006). Because of the high production and import in the European Economic Area, with annual amounts of 10 000 to 100 000 tonnes, lauric acid is in the highest category of data requirements under the REACH legislation (European Chemicals Agency 2018). Further, laurate was chosen because of the existence of reliable fish BCF data (Van Egmond et al. 1999), which could be used as a comparison and as a precursor for following investigations on other surfactants.

The equilibrium between laurate and lauric acid is dependent on the pH. The pKₐ of lauric acid was experimentally determined to be 5.0 (Smith and Anderson 1993). Thus, at a pH > 7, lauric acid is completely dissociated. The estimated log $K_{ow}$ of undissociated lauric acid is 4.7 (Gobas and Morrison 2000), whereas the log $K_{ow}$ of laurate was experimentally determined to be 3.0 (Onitsuka et al. 1989).

In contrast to most industrial chemicals, laurate is not a xenobiotic substance. It is a natural part of the physiology and lipid metabolism of any organism. The main pathways for metabolism of fatty acids are β-oxidation and biotransformation to different lipid compounds (Tolls et al. 1994; Rustan and Drevon 2005). Saturated fatty acids like lauric acid are mostly catalyzed or stored in lipids such as triacylglycerides (Sargent et al. 1995; Parrish 2009).

In the present study the bioconcentration potential of the ionic compound laurate was elucidated in H. azteca. The metabolism of the $^{14}$C-labeled test item in the freshwater amphipod as well as the fate of the compound in a flow-through and semistatic test system were investigated. The results are compared with bioconcentration data obtained in a study on laurate conducted by Van Egmond et al. (1999) with the zebrafish Danio rerio.

**MATERIAL AND METHODS**

Three bioconcentration tests with H. azteca were carried out as part of the present study. A flow-through approach (study I) and a semistatic approach (study II) were carried out to assess the bioconcentration of laurate. Subsequently, the bioconcentration of carbonate as a mineralization product of laurate was tested using a semistatic approach (study III). An overview of the test systems applied in the present studies is provided in Figure 1.

**Hyalella azteca**

The experimental animals for the bioconcentration studies were raised in the laboratory of Fraunhofer IME. Animals were kept in reconstituted water containing bromide, which is essential for H. azteca (Borgmann 1996). Culture and maintenance procedures were as described by Schlechtriem et al. (2019). Sexing of Hyalella was performed based on the presence of the large second gnathopod of males. Only mature male H. azteca with an age of approximately 3 mo were used during studies I and II, to avoid reproduction during the experiment. For study III, a mixed group including male and female amphipods was used. Animals were kept under a 16:8-h light:dark cycle with a light intensity of 800 to 900 lux in all studies.

**Chemicals**

$^{14}$C-lauric acid (carbonyl carbon-labeled), dissolved in ethanol, was obtained from Hartmann Analytics (98.4%}
radiochemical purity). Unlabeled lauric acid was obtained from Emery Oleochemicals (98.0% purity). The lipid standards for thin-layer chromatography (TLC) analysis were obtained from Sigma-Aldrich (phosphatidylincholine, trilaurin, L-α phosphatidylinositol) and Avanti (spingomyelin (d18:1/12:0), L-α phosphatidylserine, L-α phosphatidylglycerol, 19:0 cholesterol ester, cholesterol). Radiolabeled NaHCO₃ was purchased from Hartmann Analytics (99% radiochemical purity). All other chemicals were of analytical grade. A detailed list of the chemicals, materials, and instruments used can be found in Supplemental Data, Tables S1 to S3.

Preparation of the stock solutions

First, radiolabeled lauric acid was diluted with unlabeled lauric acid. After dilution, the specific radioactivity of the lauric acid was 0.0857 MBq/mg (study I) or 0.0813 MBq/mg (study II). The ethanol was evaporated at 45 °C under a stream of nitrogen. Then, an equimolar amount of solid NaOH dissolved in 30 mL ultrapure water (ultrahigh quality) was added. Lauric acid was redissolved and converted to sodium laurate at 45 °C in an ultrasonic bath until the solution was entirely clear. Stock solutions were stored at 4 °C.

For study III, radiolabeled, crystalline, sodium hydrogen carbonate (NaHCO₃) was diluted with unlabeled NaHCO₃ to obtain a stock salt with a specific activity of 0.0182 MBq/mg.

Purified (passing a charcoal filtration, aeration, and a lime stone column) copper-reduced tap water fulfilling the criteria of guideline 305 (Organisation for Economic Co-operation and Development 2012b) was used as dilution water in all tests.

Flow-through bioconcentration study (study I)

In the first bioconcentration study animals were exposed for 48 h under flow-through conditions to dissolved ¹⁴C-labeled sodium laurate, followed by a 48-h depuration phase. Because no bioconcentration studies testing ionic organic compounds in *H. azteca* were conducted before, the duration of the exposure period was chosen based on values of previous studies with non-ionic organic compounds. The application solution was created by adding the ¹⁴C-sodium laurate stock solution to 8.5 L of copper-reduced water in a brown glass bottle to reach a nominal laurate concentration of 3.0 mg/L. The solution was stirred overnight to obtain a uniform dispersion of the test item and then transferred to the test system via a mixing chamber. The application solution was diluted with copper-reduced water to reach a nominal afflux concentration of 150.0 μg/L (12.9 kBq/L) laurate. The flow was set to 6 L/h equivalent to a 3.3-h renewal period of the test medium in the 25-L glass aquarium containing 20 L of medium. The aquarium was placed in a temperature-controlled water bath (22 ± 1 °C) and stocked with 2480 male *H. azteca*. Before insertion of *H. azteca* into the test system, the flow of test medium was run for 2 d to achieve equilibrium exposure conditions. Throughout the study, the total laurate concentration in the test medium was monitored by periodic combined liquid scintillation counting (LSC) and TLC analysis. At regular intervals triplicate samples of 20 amphipods each were taken, washed with copper-reduced water, and stored at −20 °C until chemical analysis. Further, amphipods (3 × 10 animals) were collected at 0, 48, and 96 h for lipid analysis.

Semistatic bioconcentration study (study II)

During the second bioconcentration study, animals were exposed in a semistatic approach to dissolved ¹⁴C-labeled sodium laurate with a 12-h medium exchange during the uptake phase. After a 48-h uptake phase, a 48-h depuration phase followed. The test medium applied in the present study consisted of 20 L sterile filtered, copper-reduced water, which was directly spiked with the ¹⁴C-sodium laurate stock solution. An exposure concentration of 1.5 mg/L was applied, which was 10 times higher than that applied in study I, to reduce the impact of biodegradation on the relative amount of the intact test item. The test medium was stirred for 30 min for equilibration before animals (900 male *H. azteca*) were placed in the test system. The 25 L glass basin was placed in a tempered water bath (22 ± 1 °C). Throughout the study, the total laurate concentrations in the test medium were monitored by periodic combined LSC and TLC analysis. At regular intervals triplicate samples of 10 amphipods each were taken, washed with copper-reduced water, and stored at −80 °C. Further amphipods (3 × 10 animals) were collected at 0, 48, and 96 h for lipid analysis.

Bioconcentration of radiolabeled carbonate (study III)

In the third study, *H. azteca* was exposed for 48 h to ¹⁴C-sodium hydrogen carbonate (NaHCO₃) using a static test system with no medium exchange. The study was carried out at room temperature (18 ± 0.5 °C) in a 7-L glass basin containing 5 L of medium. The test basin was stocked with 900 *H. azteca*. Test medium was prepared by dissolving 73.08 mg of the radiolabeled NaHCO₃ stock salt in 5 L of copper-reduced water. The nominal radioactivity of the test medium was 265.8 kBq/L. After 32.5 h of exposure, 7.56 mg radiolabeled NaHCO₃ dissolved in 50 mL copper-reduced water, were added to the medium. Following the uptake phase, animals were transferred into a clean tank with copper-reduced water to investigate the elimination of previously accumulated radiolabeled NaHCO₃. Throughout the study, the radiolabeled carbonate concentrations in the test medium were monitored by periodic combined LSC and TLC analysis. At regular intervals triplicate samples of 10 amphipods each were taken, washed with copper-reduced water, and stored at −20 °C.

Food preparation

To reduce stress and avoid cannibalism, *H. azteca* were fed ad libitum with agar-agar cubes (DECOTABs) during all 3 studies. DECOTABs contained ground TetraMin® (Tetra) and were prepared according to Kampfraath et al. (2012) with slight modifications. In brief, 1 mL ultrahigh quality ultrapure water, 20 mg of agar, and 60 mg of TetraMin per DECOTAB were
heated, filled into a set of 1 mL cube molds, and stored at 4 °C until use. A ratio of approximately one DECOTAB (~1 g) per 200 individuals was applied per day.

**Water parameters**

In all studies, temperature, oxygen saturation, and pH of the test medium were measured daily. At the beginning and the end of each uptake and depuration phase, concentrations of ammonia, nitrite, and nitrate as well as the water hardness were measured photometrically (Nanocolor® 500D; Machery-Nagel).

**Estimation of lipid content**

Lipids of *H. azteca* collected in studies I and II were extracted following Smedes (1999) with minor modifications. Briefly, samples were homogenized in 4.5 mL of cyclohexane/isopropanol (CX/IPA) with a ratio of 5:4 (v/v). Afterward, 2.75 mL of ultrahigh quality water was added. Then, samples were vortexed, centrifuged for 12 min at 543 × g, and transferred in the organic phase into a preweighted glass vial. The procedure was repeated after adding 2.5 mL of CX/IPA (87:13, v/v) to the remaining aqueous phase. After evaporation, the lipid content was determined gravimetrically on a wet weight basis.

**Media analysis**

The aqueous concentration of the 14C-labeled test items was determined by measuring the radioactivity via LSC. Triplicates of media samples were mixed with a scintillation cocktail (Ultima Gold™ LLT; Perkin Elmer) in a ratio of 1:4 (v/v) and analyzed by a Tri-Carb 2910 TR (Perkin Elmer). In addition, the proportion of laurate and the transformed fraction in the test medium were determined by TLC analysis. Therefore, aqueous sample volumes equal to 10 Bq were directly applied to silica gel 60 F254 (Merck) plates using an automatic TLC sampler (ATS4; CAMAG). Lauric acid dissolved in ethanol or aqueous sodium laurate was used as standard. Plates were run using dichloromethane:methanol (90:10, v/v) as a mobile phase. After drying overnight under laminar flow, they were exposed to phosphor imaging plates (BAS-III; Fujifilm) for 5 d. Subsequently, plates were read by a Typhoon FLA 7000IP Bio-Imager (GE Healthcare). Recorded images were evaluated using the software AIDA Image Analyzer, Ver 3.44 (Elycia-raytest), for peak integration. At the time of medium exchange during study II, both fresh and aged media were analyzed. The time-weighted average (TWA) of the exposure concentration/radioactivity was calculated according to guideline 211 (Organisation for Economic Co-operation and Development 2012a). First (Equation 1), the area (A) under the exponential curve between each pair of subsequent measurement points was calculated using Conc 0 as the start concentration, Conc 1 as the end concentration, and the time period (t) between those pairs.

\[
A = \frac{\text{Conc} \ 0 - \text{Conc} \ 1}{\ln(\text{Conc} \ 0) - \ln(\text{Conc} \ 1)} \times t \tag{1}
\]

Then (Equation 2), the TWA concentration was calculated by dividing the total area of all periods by the total time of the uptake phase.

\[
\text{TWA} = \frac{\Sigma A}{\Sigma t} \tag{2}
\]

**Tissue analysis**

Radioactivity in *Hyalella* samples collected during studies I and III was determined by combustion of whole animals at 900 °C under O2 flow using a biological oxidizer (OX 700; Zinsser). The resulting CO2, including the radiolabeled 14C, was captured in scintillation cocktail Oxsolve C-400 (Zinsser) and analyzed in a Tri-Carb 2910 TR (Perkin Elmer). The results were used to evaluate the uptake and elimination of the test item.

*Hyalella* samples collected during study II were extracted by liquid−liquid extraction prior to combustion of the extraction residues. To allow further investigations on the metabolite pattern of the bioaccumulated material 2 extraction solvent systems were applied. The first solvent system (CX/IPA) was equivalent to the lipid extraction procedure described above (Estimation of lipid content). The second solvent system was applied as part of a modified technique introduced by Anastassiades et al. (2003), excluding the fatty acid removing cleanup. In brief, following the lipid extraction, 2 mL of acetonitrile were added to the extraction residues. Then, 2600 mg MgSO4 7H2O and 280 mg NaCl were added, and the sample was vortexed. Phases were separated by centrifuging for 12 min at 543 g, and the separated solvent phase was transferred into a glass vial. Phase separation was repeated after addition of another 2 mL of acetonitrile. The radioactivity recovered in each of the 2 solvent fractions was determined by measuring 3 subsamples with 10% of the total solvent volume by LSC using the scintillation cocktail Ultima Gold (Perkin Elmer). Finally, nonextractable residues (NERs) were determined by combustion of the remainder. Extraction methods were validated by analyzing 3 blank amphipod samples that were spiked with 50 µL 14C-sodium laurate stock solution (equal to 1.5 µg laurate with 167.9 Bq) and stored at −20 °C prior to extraction. In addition, samples of DECOTABs collected at the end of the uptake phase of all 3 studies were analyzed by combustion to investigate the extent of test item adsorption to the feed presented to the test animals. This could potentially have led to biomagnification processes, which would thus influence the results of the present study.

**Metabolite analysis**

The CX/IPA and acetonitrile extracts obtained from animals collected during study II were further analyzed for the presence of radiolabeled metabolites by applying a volume equal to 10 Bq (5 Bq for acetonitrile extracts) to a 20 × 20 cm silica gel 60 F254 plate (Merck). Samples were separated and compared to a standard of radiolabeled lauric acid. A TLC run was performed using 2 sequential solvent systems described by Bilyk et al. (1991), which was also applied by Van Egmond et al. (1999) for...
the analysis of metabolites of laurate in the tissue of D. rerio. The first mobile phase consisted of toluene:diethyl ether:ethyl acetate:acetic acid (75:10:13:1.2, v/v/v/v) and was run a distance of 8 cm from the application point. Afterward, plates were dried for 5 min under N₂ flow. The second mobile phase consisted of n-hexane:diethyl ether:formic acid (80:20:2, v/v/v) and was run a distance of 14 cm from the application point. Plates were dried for 2 d before incubation on imaging plates. Radiometric scanning was performed after 5 d (10 Bq) or 10 d (5 Bq) of incubation.

One TLC plate containing unlabeled standards of different lipid fractions (phosphatidylcholine, sphingomyelin, L-α phosphatidylinositol, L-α phosphatidylserine, L-α phosphatidyglcerol, trilaurin, cholesterol ester, and cholesterol) as single and pooled samples was run using the same solvent system. Spots were made visible by spraying with 3% (w/v) cupric acetate in 8% (v/v) phosphoric acid and charring for 15 min at 160 °C as described in Schlechtriem et al. (2004). Retention factor (RF) values of the standards were compared to the lipid fractions identified in the H. azteca extracts.

After this procedure, one representative TLC plate containing acetonitrile extracts was analyzed after a 4-cm run with ultrahigh quality water:acetonitrile:acetic acid (50:50:5, v/v/v) to screen whether the fractions stuck at the origin line consisted of one or several polar compounds.

**Decarbonation procedure**

The impact of mineralization on the presence of laurate in the test medium was elucidated by acidification of aged test medium inducing the elimination of radiolabeled carbonate as CO₂. For sample preparation, 500 mL medium were taken from study II after the first 12 h of exposure. Subsamples of this volume were analyzed by LSC, TLC, and decarbonation directly (12 h old) and after 12 h more (24 h aged) of incubation under a fume hood at room temperature. The TLC analysis was performed as described in the section Media analysis. For the decarbonation procedure, duplicates of 1 mL of each aged medium (12 and 24 h aged) were transferred into the first of 3 serially connected 20-mL vials. The setup is illustrated in Supplemental Data, Figure S1. The sample was diluted with 4 mL of ultrahigh quality ultrapure water. The subsequent vials were each filled with 5 mL of the CO₂ selective scintillation cocktail Oxsolve C-400 (Zinsser Analytic). Then, the sample in the first vial was acidified with 0.1 mL of formic acid (25%), and the system was closed quickly. Nitrogen gas was blown through the system for 5 min to elute any potentially released CO₂ into the vials containing Oxsolve. Afterward, the radioactivity of 3 different fractions was determined by LSC: radioactivity remaining in the aqueous phase, radioactivity that was volatilized and dissolved in the Oxsolve, and radioactivity recovered in acetone after the sample vial was rinsed 2 times with 5 mL of acetone.

**Autoradiographic imaging**

The spatial distribution of radiolabeled test material in Hyalella following aqueous exposure was analyzed by autoradiographic imaging. During each of the 3 studies up to 10, Hyalella were sampled for autoradiographic imaging after 48 h (end of exposure time) and 96 h (end of depuration time). The samples were shock frozen in liquid nitrogen and stored at −80 °C until analysis. In addition, control animals taken from the Hyalella culture were sampled the same way. Samples were dissected on liquid nitrogen, and cross sections as well as complete animals were exposed to a phosphor imaging plate (BAS-III; Fujifilm). After 14 d of incubation at −20 °C, the image plate was left to warm up in the dark at room temperature and read by a Typhoon FLA 7000IP Biolmager (GE Healthcare). Images were qualitatively evaluated and exported using the software AIDA Image Analyzer, Ver 3.44 (Elysia-Raytest).

**BCFs**

The steady-state BCF (BCFₜₛ) was calculated (study II) when the tissue concentrations of 3 subsequent Hyalella samplings were within ±20% of each other and not significantly different from each other. The BCFₜₛ (Equation 3) was calculated according to guideline 305 (Organisation for Economic Co-operation and Development 2012b) as the quotient of H. azteca tissue concentration/radioactivity (Cₜ) in kBq/kg and the corresponding medium concentration/radioactivity (Cₘ) in or kBq/L.

$$\text{BCF}_{\text{SS}} = \frac{C_h}{C_w}$$

In study II, also the kinetic BCF (BCFₖ) for laurate was determined. Therefore, the R package bcmfr, which is included in guideline 305 (Organisation for Economic Co-operation and Development 2012b), was applied. The simultaneous estimation of the uptake constant (k₁) and the depuration rate constant (kₑ) was based on Equations 4 and 5. These equations include the time since the start (t) and the time at the end of exposure phase (tₑ = 48 h).

$$0 < t < t_e \quad C_h = C_w \frac{k_1}{k_2} (1 - e^{-k_2 t})$$ (4)

$$t > t_e \quad C_h = C_w \frac{k_1}{k_2} (e^{-k_2 (t-t_e)} - e^{-k_2 t})$$ (5)

Finally, BCFₖ was calculated as the quotient of k₁ and kₑ (Equation 6).

$$\text{BCF}_{\text{K}} = \frac{k_1}{k_2}$$ (6)

**Lipid normalization**

A lipid normalization was applied because of the link of laurate to lipid metabolism, as well as the outcome of the autoradiographic analysis. The BCFₜₛ calculated for H. azteca was normalized to the lipid content of 5.0% for general comparability. Because no lipid contents were analyzed by Van Egmond et al. (1999), a literature search for lipid content of zebrafish was performed (Butte et al. 1991; Fox et al. 1994; Tocher et al. 2001). Consequently, BCFs were normalized to the...
lipid content of 10.2 ± 0.5% as described by Tocher et al. (2001), representing the worst-case scenario of the literature search.

**Statistical calculations**

Analysis of variance (ANOVA) was performed using Sigma-Plot 11.0 (Systat Software). Other calculations (such as BCF calculations) were performed using Excel 2016 (Microsoft). The errors of derived BCF values were calculated according to the law of error propagation (Mandel 1984).

**RESULTS**

**Flow-through bioconcentration study (study I)**

During the test period, all analyzed water parameters (oxygen saturation; pH value; concentrations of nitrate, nitrite, and ammonium; and hardness) were within the acceptable range for *H. azteca* (Supplemental Data, Table S4). The mortality of *H. azteca* during the study was 16.6% and within the normal range observed in previous studies. The lipid content of the test animals remained constant at 2.86 ± 0.15, 2.98 ± 0.28, and 2.71 ± 0.45% wet weight at 0, 48, and 96 h, respectively.

The average radioactivity of the test medium measured during the uptake phase (Figure 2, left) was 14.28 ± 0.33 kBq/L, equivalent to a laurate concentration of 166.6 ± 3.9 µg/L. A TLC analysis of aged medium samples revealed the entire radioactivity of the test medium remaining at the origin and not eluting equivalent to the sodium laurate standard. Later fate investigations with samples of study II demonstrated this radioactivity to originate from 14C-carbonate. This indicated that *Hyalella* was not exposed to the parent compound anymore. During the depuration phase, radioactivity of the medium was always below the limit of quantification. The radioactivity recovered from combusted DECOTABs collected at the end of the uptake phase was 7.7 ± 2.5 Bq/g (*n* = 2).

The radioactivity extracted from tissue samples was too low to be sufficiently quantified or further analyzed. Therefore, samples were combusted entirely. The kinetic of the radioactivity recovered from combusted *H. azteca* samples is presented in Figure 2 (right). The amount of total radioactivity measured in the tissue samples was increasing until the end of the uptake phase, to a level of 3135.4 ± 372.3 kBq/kg after 48 h. However, no steady state was reached. During the depuration phase, tissue radioactivity was slowly decreasing, and <30% of the previously accumulated radioactivity was eliminated until the end of the depuration phase lasting 48 h.

**Semistatic bioconcentration study (study II)**

During the second study, all water parameters were within the acceptable range for *H. azteca* (Supplemental Data, Table S5). The mortality of *H. azteca* during the study was 11.3%. During the study a significant increase of the lipid content in *H. azteca* (wet wt basis) was observed (Figure 3), with 1.88 ± 0.20% at 0 h, 2.95 ± 0.56% at 48 h (end of uptake), and 3.63 ± 0.32% at 96 h (end of depuration).

The radioactivity accounting for intact laurate in the uptake medium was determined by LSC and TLC (Figure 4) and remained constant at a TWA radioactivity of 115.5 ± 1.1 kBq/L (1.42 ± 0.01 mg/L). Apart from the major peak with the same RF value as the laurate standard, a minor peak was detected at the origin line, which was identified as mineralized substance. The estimated highest radioactivity originating from 14C

FIGURE 2: Total radioactivity of the exposure medium (study I) and recovered from oxidised *H. azteca* tissue (study I) as mean ± SD (*n* = 3). The dotted line indicates the switch from uptake- to depuration-phase.
carbonate (mineralized substance) was 6.5 kBq/L (5.7% of the TWA) after the first 12 h. The TWA of $^{14}$C carbonate was 2.5 ± 1.9 kBq/L. Radioactivity recovered from the combusted DECOTAB remainder collected at the end of the uptake phase was 499.2 Bq/g (n = 1).

In all analyzed tissue extracts (Supplemental Data, Figure S2), a steady increase of tissue radioactivity until the end of the uptake phase was measured, but no steady state was reached. During the depuration, none of the fractions reached complete elimination. Radioactivity in the CX/IPA extract and the NER fraction remained constant after the first 12 h of depuration, whereas the radioactivity in the acetonitrile extract decreased until the end of the study. After 48 h (end of uptake), an average amount of 71.7% of the recovered radioactivity was decreased until the end of the study. After 48 h (end of uptake), a steady increase of tissue radioactivity until the end of the study was measured, but no steady state was reached. During the depuration, none of the fractions reached complete elimination. Radioactivity in the CX/IPA extract and the NER fraction remained constant after the first 12 h of depuration, whereas the radioactivity in the acetonitrile extract decreased until the end of the study. After 48 h (end of uptake), an average amount of 71.7% of the recovered radioactivity was determined as NER. The CX/IPA and the acetonitrile extracts contained equal amounts of the recovered radioactivity, with a mean of 16.1 and 12.2%, respectively. The extraction fractions of H. azteca in comparison to D. rerio exposed to $^{14}$C-laurate (Van Egmond et al. 1999) are presented in Figure 5.

The CX/IPA and acetonitrile extracts of animals collected during the second study were analyzed for radiolabeled metabolites. Eleven different peaks (Table 1) were detected by TLC analysis of the CX/IPA extracts. Five of these peaks could be identified as polar lipid fraction, cholesterol, free fatty acids (FFAs), triacylglycerides, and fatty acid methyl esters (FAMEs). Radiolabeled polar lipids (69.5 ± 9.6% at 48 h), triacylglycerides (20.3 ± 7.3% at 48 h), and FFAs (3.5 ± 1.0% at 48 h) were detected in all samples throughout the study. Other peaks neither were consistently detected nor showed a chronological pattern (Supplemental Data, Tables S8 and S9). Peak 4 (unidentified), FAMEs, and cholesterol esters were only detected during the uptake phase. In the acetonitrile extracts, only 2 peaks were detected. The major peak was stuck at the origin line, whereas the second peak was identified as FFAs. This fraction accounted for up to 49.3% of the applied radioactivity in samples collected at the beginning of the uptake phase (3 h) and reduced to 0.0% within the first 24 h of depuration (Supplemental Data, Table S10). In an additional run of one TLC plate containing separated acetonitrile extracts using ultrahigh quality water:acetonitrile:acetic acid (50:50:5, v/v) as a solvent system, the origin peak could be split into a further 3 peaks, which were not further identified. The chronological development of radioactivity associated with polar lipids and triacylglycerides is presented in Figure 6. Radioactivity recovered in polar lipids and in triacylglycerides increased during the uptake phase to 4588.3 ± 692.9 and 1336.9 ± 463.8 kBq/kg, respectively. Although radioactivity in the polar lipid fraction decreased during depuration by approximately 40%, radioactivity continuously increased in triacylglycerides to approximately 140% of the level measured at the end of the uptake phase. The radioactivity associated with the total FFAs (Figure 7) increased until it reached a steady state between 12

![FIGURE 4: Media radioactivity of study II. The dotted lines indicate the media exchanges.](image)

**FIGURE 4:** Media radioactivity of study II. The dotted lines indicate the media exchanges.

![FIGURE 5: Exemplary comparison of the extraction fractions from Danio rerio (Van Egmond et al. 1999) and Hyalella azteca (present study) exposed to $^{14}$C laurate. NERs = nonextractable residues.](image)

**FIGURE 5:** Exemplary comparison of the extraction fractions from Danio rerio (Van Egmond et al. 1999) and Hyalella azteca (present study) exposed to $^{14}$C laurate. NERs = nonextractable residues.

![FIGURE 6: Temporal development of radioactivity associated with the main metabolites (polar lipids and triacylglycerides) of laurate in Hyalella azteca samples of study II. TAGs = triacylglycerides.](image)

**FIGURE 6:** Temporal development of radioactivity associated with the main metabolites (polar lipids and triacylglycerides) of laurate in Hyalella azteca samples of study II. TAGs = triacylglycerides.

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**TABLE 1: Retention factor (RF) values of the detected lipid fractions**

| Metabolite peaks | Minimum RF | Maximum RF | Corresponding standards |
|------------------|------------|------------|-------------------------|
| No. 1            | 0.00       | 0.00       | Polar lipids            |
| No. 2            | 0.02       | 0.03       | n.a.                    |
| No. 3            | 0.10       | 0.12       | n.a.                    |
| No. 4            | 0.16       | 0.17       | n.a.                    |
| No. 5            | 0.25       | 0.29       | Cholesterol             |
| No. 6            | 0.31       | 0.33       | n.a.                    |
| No. 7            | 0.35       | 0.39       | Free fatty acids        |
| No. 8            | 0.49       | 0.52       | n.a.                    |
| No. 9            | 0.62       | 0.64       | Triacylglycerides       |
| No. 10           | 0.67       | 0.69       | Fatty acid methylster    |
| No. 11           | 0.80       | 0.81       | Cholesterol ester       |

*Minimum and maximum RF values of all analyzed samples are shown. Peaks that matched the applied standards are assigned in the column “Corresponding Standards.” The RF values of the standards are presented in Supplemental Data, Table S7. n.a. = not applicable.*
and 30 h with 495.4 ± 91.9 kBq/kg (ANOVA, not significant). Afterward, the FFA-associated radioactivity started to decrease by approximately 30% until the end of the uptake phase. During the depuration phase, radioactivity further decreased to a level of 14% of the previously accumulated radioactivity.

Bioconcentration of FFAs

The uptake and depuration kinetics of laurate, measured as FFAs, is presented in Figure 7. It cannot be excluded that the FFA fraction identified by TLC analysis contained further elongation or desaturation products apart from lauric acid. A BCF_{SSL} of 4.3 ± 0.9 was calculated for laurate in H. azteca, and kinetic BCF estimates (Supplemental Data, Table S11) were within a similar range (5.9 ± 1.7). No lipid content was measured by Van Egmond et al. (1999); thus, a lipid content of 10.2 ± 0.5 (Tocher et al. 2001) was assumed for the lipid normalization as a worst-case scenario based on literature values (Butte et al. 1991; Fox et al. 1994; Tocher et al. 2001). For the lipid content at steady state (24 h), the mean lipid content of the 0- and 48-h samples (2.42 ± 0.69%) was used. The 5.0% lipid-normalized steady-state BCF (BCF_{SSL}) of laurate was 8.9 ± 3.1. The BCF_{SSL} based on 10.2% lipid content was 18.1 ± 6.3, and the corresponding lipid-normalized kinetic BCF (BCF_{KL}) was 22.7 ± 7.5. A comparison of the BCF values calculated for H. azteca with the BCF value estimated by Van Egmond et al. (1999) is presented in Figure 8.

Decarbonation procedure

During the uptake phase of study II, TLC analysis of the aged medium collected for decarbonation revealed that 3.6% of the radioactivity of the 12-h aged medium and 100.0% of the 24-h aged medium remained at the origin. The remaining 96.4% radioactivity of the 12-h aged sample was recovered in a single peak with the same RF value as the laurate standard. The total recovery of radioactivity after decarbonation was 91.4 ± 1.8 and 99.4 ± 1.8% from the 12- and the 24-h aged media, respectively. The recovered radioactivity in the 3 different fractions is illustrated in Figure 9. Radioactivity of the 12-h aged samples was almost only recovered from the water (54.6 ± 5.9%) and acetone (35.7 ± 5.1%) fraction, whereas radioactivity of the 24-h aged medium was mostly recovered in the Oxysolve fraction (91.6 ± 1.3%). An amount of 7.5 ± 0.6% radioactivity remained in the aqueous phase of the 24-h aged samples. Recoveries in the second Oxysolve vial were always <0.8%, demonstrating a negligible loss of the system. The pH of the acidified samples was between 2.5 and 2.6.

Bioconcentration of radiolabeled carbonate (study III)

All water parameters were within the acceptable range for H. azteca (Supplemental Data, Table S12). The mortality of H. azteca during the study was 7.6%.

The calculated TWA of the radioactivity in the test medium was 228.6 ± 10.7 kBq/L (Figure 10, left). Media samples applied to TLC analysis using the same solvent system as described showed that radioactivity remained entirely at the origin line. Radioactivity recovered from combusted DECOTAB remains collected at the end of the uptake phase was 79.0 Bq/g (n = 1).

The radioactivity recovered from H. azteca samples collected during the third study is presented in Figure 10 (right). No steady state was reached at the end of the uptake phase with a radioactivity of 14281.2 ± 3791.2 kBq/kg. During depuration, radioactivity decreased to 34% of the previously accumulated amount but remained constant at this level after the first 24 h.
 Autoradiographic imaging

Animals collected from all 3 studies were analyzed by autoradiographic imaging. Radioactivity was recovered in 5 compartments of *H. azteca* cross sections: exoskeleton, protocerebrum (nervous system), lipid drops, gut, and diffuse radioactivity distributed in the entire animal. Further, radioactivity within the cross section was compared to radioactivity attached to the outer surface of the amphipods. A distinct pattern for samples of the different studies was observed (Supplemental Data, Table S13). Representative autoradiograms of cross sections from samples taken at the end of the uptake phase of all 3 studies are presented in Figure 11. No radioactivity was observed in autoradiograms of the control samples. In all other autoradiograms, the exoskeleton of *H. azteca* could be clearly differentiated against the background. The protocerebrum was distinguishable in all samples except for samples taken at the end of depuration of study I. In studies I and II the entire cross section showed a diffuse distribution of radioactivity, whereas the radioactivity in samples of study III was mostly found in the sections, along the exoskeleton, and aggregated at the protocerebrum. The outer surfaces of samples from studies I and III were blackened more intensely in the autoradiographic images than the cross sections, but the resulting contrast was similar to samples of study II. Further, lipid droplets in *Hyalella* were visible in all autoradiograms from study II and in some samples taken at the end of the uptake phase of study I. Only at the end of the uptake phase of study II was radioactivity obviously present in the gut of single *Hyalella*.

**DISCUSSION**

The performance of the 3 bioconcentration studies provided a significant insight into the fate of dissolved laurate in the flow-through and semistatic test systems and the bioconcentration behavior of the FFA in *H. azteca* compared to *D. rerio*.

Mineralization of laurate in the test system

The application of the test item under flow-through conditions (study I) did not guarantee a stable exposure concentration of the parent test item, even though the total radioactivity of the medium remained stable. The TLC analyses of the test medium revealed that the entire radioactivity remained at the origin line of the TLC plate in contrast to the laurate standard. The results indicated mineralization as the major transformation pathway. Fatty acids are a rich source of energy for microbial growth. Fatty acids are oxidized in the β-oxidation pathway after conversion to coenzyme A (CoA) esters. In this cyclic pathway, fatty acids are degraded to acetyl-CoA, which can be fed into the tricarboxylic acid cycle or used in biosynthesis (Prescott et al. 2002). The mineralization of laurate was confirmed in study II by acidification of the 24-h
aged test medium leading to CO₂ elimination, whereas intact laurate remained in the water phase or attached to the glass walls and was removed by acetone. A fraction of 7.5 ± 0.6% remaining in the acidified aqueous phase of the 24-h aged samples could indicate either the minor presence of a polar intermediate product or that further incubation under N₂ flow would have been required to reach a complete CO₂ elimination. Because of the high pH of the test medium (>8) the ¹⁴CO₂ formed during the studies remained in the test medium as dissolved carbonates (Wojtowicz 1995), resulting in a stable total radioactivity in the test medium. Biodegradation was also mentioned by Van Egmond et al. (1999) as an important issue potentially affecting the performance of fish bioconcentration studies with laurate. However, in the study with D. rerio, the impact of biodegradation was minimized by a high flow rate (one volume replacement every 28 min), which was not applicable for the H. azteca test system in the present case.

Bioaccumulation of radiolabeled carbonate in H. azteca

Almost no radioactivity was extractable from H. azteca samples collected during the first flow-through study (study I). However, a significant amount of radioactivity was recovered after combustion of the residues. In addition, the results of the tissue analysis did not follow the usual first-order bioconcentration kinetics and were rather similar to the results obtained during the carbonate uptake study (study III). The results strongly suggest that mineralized laurate was accumulated in the calcified exoskeleton of H. azteca as hydrogen carbonate (HCO₃⁻). This was confirmed by autoradiographic imaging of test animals exposed to radiolabeled carbonate. Although the role of calcium in the calcification process of crustaceans has been well explored for economically important species (Cameron and Wood 1985; Hirotoshi et al. 2004; Jasmani et al. 2008), less knowledge exists about the mechanisms involved in the carbonate uptake. It is suggested that both intercellular and extracellular carbonic anhydrases play a major role in the calcification of crustaceans (Roer and Dillaman 1984; Home et al. 2002; Lavalli and Spanier 2007; Jasmani et al. 2008).

Uptake of radiolabeled laurate by H. azteca

In contrast to the flow-through system, stable exposure conditions were obtained in the semistatic test system (study II). Medium replacements were carried out every 12 h to minimize microbial growth causing degradation of the test item. The limited flow of the flow-through system was insufficient at removing the rapidly developing and adapting microbial community. These results do not necessarily prove a general impossibility of maintaining a stable laurate exposure concentration using a flow-through system with Hyalella. However, in the present case the semistatic design proved to be more robust. Further, this saved resources and radioactive material and improved water disposal handling. The bioavailability of dissolved laurate could be impaired by adsorption of the test item to the food and the formation/precipitation of particles such as calcium laurate. However, as shown in study II, the radioactivity of test items adsorbed to the feed was low. Further, the chosen medium concentration (1.5 mg/L) and hardness (1.1 mmol/L) in the test system were in a range where the formation of calcium laurate can almost be excluded, as demonstrated by Van Egmond et al. (1999).

 Autoradiographic imaging showed that a different pattern of spatial distribution was observed for H. azteca collected from the second study compared to studies I and III. The distribution of radioactivity was obviously highly associated with lipid drops, the protocerebrum (nervous system), and sometimes the gut but was also found in high amounts in the exoskeleton and at low levels throughout the animal. In a previous study on the uptake of ¹⁴C-triellate (log KOW 4.6) in Hyalella, the nervous system and lipid storage were also identified as the major accumulation compartment of the lipophilic compound (Arts et al. 1995). The spatial distribution of laurate can be explained by the compound being metabolized, for instance, to cholesterol, which is abundant in the nervous system, or bound to different lipid compounds such as triacylglycerides, which are the major component of lipid drops. The radioactivity deposited in the exoskeleton of H. azteca was not observed by Arts et al. (1995) and thus strongly supports the previous presumptions on the incorporation of ¹⁴C from mineralized laurate into calcified structures. Apart from the exoskeleton, crystalline structures are also typical for the eyes of insects and crustaceans (Hallberg et al. 1980). This may explain why also in the bioconcentration study with radiolabeled carbonate (study III) a strong blackening was observed in the protocerebrum area during autoradiographic imaging, attributable to the close connection with the eye section. However, apart from the uptake of carbonate from the test medium, formation of radiolabeled carbonate could also occur as the result of the animals’ metabolism following uptake of ¹⁴C-laurate. This may explain similarities between the NER fractions and autoradiographic images obtained for Hyalella which were exposed to dissolved ¹⁴C-laurate (study II) or carbonate (studies I and III). Both carbonate uptake pathways were described for crustaceans by Greenaway (1974), Cameron and Wood (1985), and Wheatly and Gannon (1995). The diffuse radioactivity observed in autoradiographic images of Hyalella cross sections collected in studies I and II may be explained by incorporated ¹⁴C-laurate in the polar lipids of membranes. Radioactivity in the area of the gastrointestinal tract as observed in a single animal collected from study II may have been caused by the incomplete removal of ¹⁴C-laurate attached to the ingested diet (DECOBATs).

Metabolism of laurate in H. azteca

Eleven different metabolites were observed in the CX/IPA extracts from animals collected during study II. The broad variety of observed biotransformation products may be explained by the fact that lauric acid is not a xenobiotic compound. Fatty acids play a key role in the lipid metabolism of all organisms. Fatty acids can function as metabolic fuel, are found in membranes, and can act as gene regulators (Rustan and Drevon 2005). Lauric acid can thus be transformed by a variety of metabolic pathways. Because polar lipids and fractions of the acetonitrile extract were not further separated, a higher
number of laurate metabolites as described in the present study is likely for *H. azteca*.

Radiolabeled polar lipids (69.5 ± 9.6% at 48 h) and triacylglycerides (20.3 ± 7.3% at 48 h) accounted for the major proportion of radioactivity extracted from *H. azteca*. Polar lipids consisting of different phospholipids (L-α phosphatidylcholine) such as phosphatidyl choline are important components of the cell membranes, whereas triacylglycerides represent the central energy storage. In the CX/IPA extract of *H. azteca*, the FFA fraction (3.5 ± 1.0% at 48 h) thus contained only a minor proportion of radioactivity. Biochemically, triacylglycerides and L-α phosphatidylcholine are related because they both possess a glycerol backbone to which 2 or 3 fatty acids are esterified. Therefore, the question remains whether laurate that is simply bound to the major lipid classes should also be taken into account for bioaccumulation assessment. These fatty acids can be easily released from the parent lipid classes, for example, for fatty acid analysis (Schlechtriem et al. 2008b).

Cholesterol is formed via the mevalonate pathway, serves as a precursor for vitamin D and steroids, and supports membrane stability (Goldstein and Brown 1990). The radioactivity detected in the present study in cholesterol suggests that the 14C could have entered cholesterol synthesis via 14C CoA derived from the catabolism of radiolabeled laurate. This indicates that radioactivity derived from radiolabeled laurate could have potentially ended up in a broad variety of metabolites following catabolization of the parent compound.

In the second study, a decrease of the FFA fraction in *H. azteca* was already observed during the uptake phase, which may indicate metabolic adaptation to the test item. Similar effects have been observed in fish bioconcentration studies on xenobiotic compounds (Schlechtriem et al. 2017). Free fatty acids are surface active compounds which can potentially impair membrane integrity (Fluhr et al. 2001). The increased biotransformation of the test item may have been induced by the test animals to counteract this process.

The strong increase of lipid content in *H. azteca* during the second bioconcentration test, almost doubling from the beginning (1.88% wet wt) to the end of the study (3.63% wet wt), was not observed in previous studies with *H. azteca* using the same feed (Schlechtriem et al. unpublished data). The lipid content of *H. azteca*, which was previously exposed to dissolved laurate, further increased even during the depuration phase. However, the mass of the accumulated laurate was far too low to explain the observed increase in lipid weight. Crustaceans have an absolute requirement for essential fatty acids (EFAs) such as eicosapentaenoic acid (20:5n-3) and have evolved effective strategies to store and conserve such EFAs (Schlechtriem et al. 2006, 2008a). Deficiencies in EFAs can be related to a limited quantitative supply or a nutritionally inadequate EFA composition of the diet. As a consequence of inadequate dietary fatty acid composition, growth and reproduction can be impaired. Brett et al. (2009) showed in feeding studies with the crustacean *Daphnia magna* that terrestrial particulate organic matter mostly containing saturated fatty acids clearly reduced the nutritional value of the diet. In the present study, bioconcentration of laurate may have induced a gradual displacement of EFAs in the animal tissue, leading to an inadequate representation of these compounds of high physiological importance. It thus appears likely that the uptake of laurate might have promoted the dietary uptake and storage of lipids originating from the food (DECOTABs) containing essential highly unsaturated fatty acids attributable to the high content of fish food (TetraMin). In this way the test animals may have taken care of their absolute requirement of EFAs but at the cost of an increased lipid content. Further research is required to elucidate the bioconcentration of dissolved fatty acids in aquatic organisms and potential effects on key physiological processes.

**BCFs**

The BCF (5.0% lipid-normalized, 8.9 ± 3.1; 10.2% lipid-normalized, 18.1 ± 6.3) obtained in the present study for laurate (FFA) was lower than the BCF calculated for the same test compound in Van Egmond et al. (1999) for *D. rerio* (BCF 255 ± 22). The BCF estimations of both studies were based on the parent compound only, which accounted for a minor fraction of the total radioactivity. Thus, the BCF determinations of the 2 studies can be seen as comparable. The result obtained for this ionic compound differs from the results of former investigations on lipid-accumulating compounds where BCF values calculated for *H. azteca* always tended to be higher compared to those of fish (Schlechtriem et al. 2019). However, from a regulatory point of view, both studies arrived at a comparable result, with the BCF values calculated for fish and *Hyalella* being far below the regulatory threshold for B assessment (BCF = 2000) applied in the PBT classification of chemical substances under the European REACH Regulation (European Commission 2011).

Radioactivity distribution in *H. azteca* showed that calculation of BCF values based on total radioactivity should be carried out with caution. In the fish bioconcentration study with *D. rerio* (Van Egmond et al. 1999), only a small amount of radioactivity was recovered by combustion of extracted residues, whereas radiolabeled NERs represented a high proportion (>70%) of the total radioactivity in *H. azteca*. Calculation of a BCF for 14C-laurate based on total accumulated radiolabel (including the total of the parent substance, any retained metabolites, and the high amount of assimilated carbon) would have greatly exceeded the *Hyalella* BCF calculated for extracted laurate (FFA). On the other hand, measurement of only total radioactivity of the test medium could lead to an underestimation of the calculated BCF, for instance, because of lower exposure concentrations attributable to biodegradation of the test item, as shown in the present study. If total radioactive residues are measured alone in *Hyalella* BCF studies (e.g., by combustion or LSC), separation procedures such as TLC should be thus applied to calculate BCF values which are directly comparable to a BCF derived by specific chemical analysis of the parent substance.

**CONCLUSIONS**

An alternative test setup for bioconcentration studies with organic, neutral compounds using the freshwater amphipod

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H. azteca was recently suggested. The present study has shown that the test system is also suitable for testing ionic organic compounds, but in the present case adjustments (semistatic approach) of the test system were necessary to reduce the rapid biodegradation of the test item. The bioconcentration mechanisms of laurate were elucidated, confirming the low bioaccumulation potential of the test item previously observed in fish. However, more organic ions with various properties need to be tested to assess whether, apart from the correlation results of the 2 test systems also exists for such compounds. A test strategy is required to integrate both tests into a coherent testing and assessment strategy.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4623.

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Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (christian.schlechtriem@ime.fraunhofer.de).

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