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CAN SOLID-PHASE MICROEXTRACTION REPLACE SOLVENT EXTRACTION FOR WATER ANALYSIS IN FISH BIOCONCENTRATION STUDIES WITH HIGHLY HYDROPHOBIC ORGANIC CHEMICALS?

LEONARD BÖHM,[a,*] ROLF-ALEXANDER DÜRING,[a] HANS-JÖRG BRUCKERT,[b] and CHRISTIAN SCHLECHTRIEM[b]
[a]Institute of Soil Science and Soil Conservation, Research Center for BioSystems, Land Use and Nutrition (iFZ), Justus Liebig University Giessen, Giessen, Germany
[b]Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Schmallenberg, Germany

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Abstract: With the aim to refine water analysis in fish bioconcentration studies, automated solid-phase microextraction (SPME) was used as an alternative approach to conventional solvent extraction (liquid–liquid extraction [LLE]) for the extraction of 3 hydrophobic organic chemicals (HOCs; log K_{OW} 5.5–7.8) from flow-through studies with rainbow trout (*Oncorhynchus mykiss*). The results showed that total concentrations extracted by SPME combined with internal standards and LLE are equal. The results further verify the possibility of simultaneous extraction of total and freely dissolved HOC concentrations by SPME. Freely dissolved concentrations allow the assessment of sorption and bioavailability of HOCs in bioconcentration studies and their potential impact on resulting bioconcentration factors (BCFs). Reduction in freely dissolved water concentrations can result in an underestimation of BCFs if they are calculated based on total water concentrations. For polychlorinated biphenyl (PCB) 153, a significant increase in BCF value was observed when freely dissolved concentrations were taken into account. However, log BCF values calculated based on freely dissolved concentrations did not correlate linearly with log K_{OW} values above 5 to 6. This pointed to further influences besides a reduction in freely dissolved water concentrations by sorption to organic matter. The results can aid in assessment of the factors that influence bioconcentration systems and also give important information regarding the possible replacement of LLE by SPME for water analysis of highly HOCs in fish bioconcentration studies. *Environ Toxicol Chem* 2017;36:2887–2894. © 2017 The Authors. Environmental Toxicology and Chemistry Published by Wiley Periodicals, Inc. on behalf of SETAC.

Keywords: OECD TG 305 Bioaccumulation Rainbow trout (*Oncorhynchus mykiss*) Bioconcentration factor (BCF) Adsorption Water quality guidelines Organic matter

INTRODUCTION

Fish bioconcentration studies are needed for the safety assessment of chemicals as well as for the understanding of processes regarding the interaction of organisms with their environment. Such studies are commonly performed according to guideline 305 of the Organisation for Economic Co-operation and Development (OECD) [1]. The outcome is a bioconcentration factor (BCF), which is calculated by the chemical's concentration in fish (C_f) divided by its concentration in the water phase (C_w). The guideline does not prescribe specific methods for extraction of the water phase. The method most often used is liquid–liquid extraction (LLE) [2–5], which is considered an exhaustive extraction method yielding total water concentrations ($C_{w,t}$). The LLE procedure is time-consuming and uses large amounts of organic solvents, which is contrary to several of the 12 principles of green chemistry [6,7]. Bioaccumulation of neutral organic chemicals is of regulatory relevance for substances with log *n*-octanol–water partition coefficients ($\log K_{OW}$) > 3 and as a general rule increases with increasing hydrophobicity. However, for substances with log K_{OW} > 5 to 6, the correlation of BCF and K_{OW} values levels off or even declines. The parameters that are assumed to cause this leveling off (sometimes referred to as hydrophobicity cutoff) are physiological as well as analytical/artificial processes. Such parameters include restricted membrane permeability because of the molecular size and weight of test substances, elimination into feces, *n*-octanol as an inappropriate substitute for membrane lipids, and sorption to organic matter in the test system [8–14]. Highly hydrophobic organic chemicals (HOCs) can be sorbed to organic matter in the test system, leading to a reduction in freely dissolved water concentrations ($C_{w,f}$), which are considered to be the concentrations bioavailable for fish [15–18]. If $C_{w,f}$ is reduced by sorption to organic matter in a test system but $C_{w,t}$ is extracted, an underestimation of BCF values compared with systems without organic matter impact is possible [13,19]. However, the organic matter content in test systems, which is quantified by the total organic carbon (TOC) concentration, is often not specified or even investigated. This can lead to a high variation in BCF data for highly HOCs [19]. In contrast to LLE, $C_{w,f}$ can be determined by solid-phase microextraction (SPME), a nonexhaustive extraction method based on partitioning kinetics of a chemical between the sample and a coated fiber, which is introduced directly to the sample or in the headspace above the sample [20–25]. This technique can be used under both nonequilibrium and equilibrium conditions. Extraction by fibers can be performed manually or can be automated with an autosampler. For highly HOCs, the time until equilibrium between fiber and analytes is reached can be several days. Therefore, parallel extraction of samples requires the use of several single fibers. After equilibrium is reached, the fibers

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* Address correspondence to leonard.boehm@umwelt.uni-giessen.de

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can be extracted either by solvents [13,26] or by thermal desorption in the injector of a gas chromatography-coupled to a mass spectrometer (GC–MS). This requires manual labor unless fibers are stored and automatically introduced to the injector by an automated liner exchange system [27]. For the quantification, analyte-specific fiber–water partitioning coefficients are needed [26,28–30]. In small sample volumes, the depletion of analytes by the fiber is a relevant issue because it can alter the ratio of freely dissolved and organic matter-bound analytes when analytes desorb from the organic matter in the sample to form a new equilibrium with fiber and aqueous solution [31,32]. Because the analyte uptake by the fiber is proportional to the freely dissolved analyte concentration, extraction can just as well be performed in the kinetic phase of the equilibration process, that is, under nonequilibrium conditions. In this way, extraction times can be reduced to 20 to 60 min, depending on the analyte concentration in the sample and the fiber type used [33]. Under nonequilibrium conditions, the extracted concentration can be affected by an accelerated transport of the analyte by dissolved organic matter in the stagnant water layer around the fiber [32]. This effect can be reduced, for example, by agitation of sample and fiber. Because time and temperature greatly affect the amount of analyte extracted by the fiber, SPME-compatible equipment of the autosampler is advisable. Because of the short extraction times, samples can be extracted consecutively with the same fiber. Quantification is then performed simply by external calibration with samples that contain the analytes in aqueous solution. With automated SPME, the fiber is directly transferred to the injection system of the GC–MS, where the analytes are thermally desorbed. Further comprehensive information on SPME can be found, for example, in the *Handbook of Solid Phase Microextraction* [34]. While SPME is now recommended for highly HOCs in the revised OECD guideline 305 [1], its use is still not commonplace. In addition to reluctance to overturn well-established routines, a major reason hampering the replacement of LLE by SPME is the concern that data from both methods might not be comparable. The comparability of results from alternative methods with results obtained from long-time applied methods is an important prerequisite, for example, for regulation. However, while LLE only provides the measurement of $C_{w,t}$, the benefit of automated SPME could be the simultaneous determination of $C_{w,t}$ and $C_{w,f}$ [35]. While only freely dissolved molecules bind to the fiber yielding $C_{w,f}$ [16,32,36], the addition of an internal standard with similar characteristics as the test substance allows the determination of $C_{w,t}$ when the amount of internal standard, which is sorbed to organic matter in the sample, is taken into account [35,37]. This would ensure the comparability of data from alternative and conventional methods. Furthermore, more reliable and precise data would be obtained facilitating the interpretation of conditions and mechanisms/processes in the test system. Therefore, the purpose of the present study was to examine the use of automated SPME in bioconcentration studies and to assess a possible replacement of LLE by SPME. Therefore, the extraction of water phases from flow-through bioconcentration studies according to OECD guideline 305 was investigated by SPME and compared with data from LLE [38]. The bioconcentration studies were performed with rainbow trout (*Oncorhynchus mykiss*) and 3 HOCs (hexachlorobenzene [HCB], o-terphenyl [OTP], and polychlorinated biphenyl [PCB] 153), which cover a broad range of hydrophobicity (log K_{OW} 5.5–7.8). The objectives of the present study were 1) to verify that extraction of the aqueous phase in fish bioconcentration studies by SPME and LLE leads to comparable results in $C_{w,t}$, 2) to investigate whether $C_{w,t}$ and $C_{w,f}$ can be obtained simultaneously by automated SPME for very low C_w of highly HOCs, and 3) to examine the possible impact of the extraction method on BCF values.

MATERIALS AND METHODS

Chemicals

Hexachlorobenzene and OTP were purchased from Sigma-Aldrich. Both PCB 153 and the internal standards HCB-$^{13}C_6$, and PCB 138 were purchased from Dr. Ehrenstorfer, and OTP-d_{14} from CDN Isotopes. Stock solutions of the substances (all $\geq$99% purity) were prepared in methanol ($\geq$99%, p.a., Sigma-Aldrich). Because of similar physicochemical characteristics, PCB 138 was used as internal standard for PCB 153 instead of an isotopologue. Molecular structures of the substances are given in the Supplemental Data, Figure S1, and physicochemical properties are given in the Supplemental Data, Table S1.

BCF studies with rainbow trout

The BCF studies were conducted as described in detail in Schlechtriem et al. [38]. Briefly, 2 flow-through fish bioconcentration studies were performed with juvenile rainbow trout (3.3 ± 0.4 g in the first and 3.8 ± 0.4 g for the second study) according to OECD guideline 305. Growth rate constants were 0.0201 and 0.0226 d^{-1} for the first and second studies, respectively. The lipid content of approximately 5 and 6% at the beginning of the uptake period increased to approximately 6 and 7 to 8% in the first and second studies, respectively. The HOCs applied were HCB and OTP in the first study, and PCB 153 and dibenz[*a,h*]anthracene (DBA) in the second study. Because of an unstable supply of C_w of DBA during the second study and its strong metabolism in *O. mykiss* [38], no valid time-weighted average concentrations could be derived from SPME analysis (fewer sampling days compared with LLE resulted in an incorrect weighting of extremely increased DBA supply), and no BCF could be estimated for DBA, which is thus not considered in the present study. Substances were applied solventless as column-generated concentrations. As test vessels, 100-L glass aquaria each filled with 70 L of purified water were used for control and treatment in both studies. A continuous flow of test media into the experimental tanks was maintained throughout the first and second study, approximately 22 and 21 L h^{-1}, respectively, equivalent to 7.5 and 7.2 volume replacements per day, respectively. The experimental tanks were stocked with 70 and 68 fish in the first and the second study, respectively. Fish were fed with commercial feed for juvenile rainbow trout (Biomar, Inicio Plus 0.8 mm) with a daily ration of 1.5% of their body weight. With regard to a potential impact of organic matter on the bioavailability of test substances (e.g., from fish feed) [18], tanks were carefully cleaned every day 30 to 60 min after feeding, as stated in OECD guideline 305. The organic matter in the water phase was determined weekly by quantifying TOC as the sum of particulate organic carbon (POC) and dissolved organic carbon (DOC; Supplemental Data, Figures S2 and S3). The uptake period was 56 d in both studies followed by a depuration period lasting 28 and 56 d for the first and second study, respectively. Fish were extracted by accelerated solvent extraction with acetone and dichloromethane (1:1, v/v) followed by cleanup, concentration, and measurement of extracts by GC–MS. Homogenates from unexposed fish were

spiked with 7 different test substance concentrations and extracted accordingly, yielding a matrix calibration that was used within quantification to prevent an underestimation of tissue concentrations. Tissue concentrations at the end of the uptake phase were 11.2 mg kg^{-1} for HCB, 4.6 mg kg^{-1} for OTP, and 293 μg kg^{-1} for PCB 153. Experimental conditions and results of the fish studies (solid-phase desorption dosing system, flow-through fish test conditions, chemical and lipid analysis of fish, growth performance, concentrations in fish during uptake and elimination periods, uptake and elimination rates, different BCF estimates) are comprehensively discussed in Schlechtriem et al. [38].

Analysis of water samples

Water concentrations were determined regularly throughout the uptake period of both studies by LLE and SPME. The LLE of water samples is described in Schlechtriem et al. [38]. Samples for water analysis following SPME were collected from the test vessels on days 1 to 5, 8, 12, 15, 19, 22, 26, 29, 34, 36, 40, 43, 47 to 49, and 54 ($n = 4$, each) of the uptake phase in the first study and on days 6, 27, 34, 44, 48, 51, and 55 ($n = 5$–8, each) of the uptake phase in the second study. For SPME analysis, 20-mL aliquots from the test vessels were transferred to brown glass vials (20-mL headspace vials with magnetic caps, CS Chromatographie Service) and immediately spiked with the respective internal standards (Supplemental Data, Figure S1). During the first study, samples were collected and prepared twice on days 5, 12, 19, 26, 34, 40, 47, and 54 ($n = 4$, each) for a comparative measurement in a second laboratory. Samples were measured within 1 d after sampling in laboratory 1. The samples for laboratory 2 were packed in extruded polystyrene foam boxes together with ice packs and were transferred within 2 d by courier mail. During the second study, samples were only measured in laboratory 2. Samples were measured in both laboratories by automated SPME in immersed mode. Actual depletion of analytes by the fiber is given in the Supplemental Data, Table S2. Water samples and external calibration samples (first study/second study) were thermally equilibrated (5 min) prior to extraction (30 min/60 min) with polydimethylsiloxane (PDMS) fibers (100 μm/7 μm; Supelco). Samples were agitated (250 rpm) and heated (30 °C) during equilibration and extraction in a heating device within the autosampler. Subsequently, fibers were thermally desorbed for 3 min in the GC injector followed by GC–MS for separation and detection of analytes. Before sample extraction, fibers were cleaned in a separate heating device (280 °C for 8 min/320 °C for 10 min) to prevent carry-over of analyte residues on the fiber. Further details on instruments and methods as well as on quality assurance and quality control are given in the Supplemental Data, Tables S3 to S6.

Calculation of water concentrations

The $C_{w,f}$ values from SPME were calculated directly from the GC–MS signals (peak area, A) of water samples and external calibration samples. With SPME, only the freely dissolved HOC molecules are extracted (see *Introduction* section). Hence, $C_{w,f}$ of samples is calculated from the peak areas of water samples and a calibration curve from external calibration samples. External calibration samples were analyzed together with the water samples under the same conditions. The calibration curve was calculated as a linear regression line made up as a function of the peak areas of external calibration samples plotted against the nominal concentrations of external calibration samples. For the calculation of $C_{w,f}$, the internal standards remained unconsidered. The $C_{w,t}$ values from SPME were calculated according to the calculation of $C_{w,f}$, except that calculation was performed after correction of the peak areas by internal standards. Compared with the extracted amount of the internal standards in the external calibration samples, the extracted amount of the internal standards in the water samples is reduced by sorption to organic matter. Reduction of the internal standard by sorption is in the same dimension as the reduction of the test substance by sorption when an internal standard with similar characteristics as the test substance is used (see *Introduction* section). Therefore, correction of peak areas for the calculation of $C_{w,t}$ was performed according to

$$cA_{ts,s} = A_{ts,s} \times mA_{is,cs}/A_{is,s} \quad (1)$$

where $cA_{ts,s}$ is the corrected area of the test substance in a specific sample, $A_{ts,s}$ is the uncorrected area of the test substance in this specific sample, $mA_{is,cs}$ is the mean area of the internal standard in the calibration samples, and $A_{is,s}$ is the area of the internal standard in the specific sample. The C_w values were calculated as time-weighted average concentrations. For this, weighted average concentrations were calculated by multiplying the average of measured concentrations from 2 consecutive sampling days by the time period (h) between both sampling days. All weighted average concentrations were then summed up and divided by the total time (h) of the uptake period [38]. Calculation of water concentrations was done independently in both laboratories to allow an interlaboratory comparison. Results were compared only at the end of both studies. A comparison of means for C_w values derived from SPME and LLE was carried out by *t* test (SPSS 24).

Calculation of BCFs

The BCF values were calculated on a fresh weight basis according to

$$BCF_{ss} = C_f/C_w \quad (2)$$

as the ratio of the substance concentration in fish (C_f, μg kg^{-1}) and the time-weighted average concentration in the water phase (C_w, μg L^{-1}) assuming steady state ($_{ss}$) [1]. To check for a possible impact of the analytical method used for measuring C_w on the result of BCF studies, BCFs were calculated based on $C_{w,t}$ and $C_{w,f}$ from SPME measurements and compared with the BCF values calculated based on $C_{w,t}$ from LLE ($C_{w,t\ (LLE)}$; Supplemental Data, Table S7). Because steady state of the test substances in the fish tissue was not reached within the uptake phase of 56 d [38], direct calculation of BCF_{ss} values was not possible. However, BCF calculation according to Equation 2 is beneficial when comparing potential analytical influences on C_w and further on BCF. Therefore, the apparent concentration in fish at steady state ($C_{f,app}$; Supplemental Data, Table S8) was used for the calculation of BCF values according to Equation 2. The $C_{f,app}$ value was derived according to

$$C_{f,app} = BCF_k \times C_{w,t(LLE)} \quad (3)$$

from the kinetic BCF values (BCF_k; Supplemental Data, Table S7) and $C_{w,t\ (LLE)}$ from Schlechtriem et al. [38]. Because BCF_k values are calculated from the ratio of the uptake rate constant k_1 and the depuration rate constant k_2, they are considered to reflect conditions in steady state, that is, BCF_k is considered to be equal to BCF_{ss} [1]. The BCF_k values were

calculated with the R-based software package bcmfR (Ver 0.3-2) according to the upcoming guidance document for OECD guideline 305 [39]. As recommended there, BCF_k values were calculated with the simultaneous fitting of uptake and depuration phases, instead of the sequential procedure applied in Schlechtriem et al. [38]. The R-package and the instructions on how to use it are made available by the OECD. The best fit was achieved without data transformation for all of the 3 test substances. The BCF_k values as well as uptake and depuration curves are given in the Supplemental Data, Table S7 and Figure S4.

RESULTS AND DISCUSSION

$C_{w,t}$ derived from SPME analysis

The $C_{w,t}$ results from SPME analyses were equal to results from LLE in both studies. In the first study, time-weighted average $C_{w,t}$ values of HCB and OTP measured by SPME were $0.40 \pm 0.04\ \mu g\ L^{-1}$ (standard deviation [SD]) and $0.43 \pm 0.05\ \mu g\ L^{-1}$, respectively. These were in the same range of $C_{w,t}$ from LLE of $0.39 \pm 0.03\ \mu g\ L^{-1}$ and $0.45 \pm 0.04\ \mu g\ L^{-1}$, respectively (Figure 1 and Supplemental Data, Figures S5 and S6). A comparison of means by *t* test confirmed that differences were not significant ($p = 0.428$ for HCB, $p = 0.098$ for OTP). Differences between concentrations were mostly higher over time than between SPME and LLE. For the highly hydrophobic PCB 153, SPME and LLE yielded equal results as well ($p = 0.544$). The time-weighted average $C_{w,t}$ was $23 \pm 4\ ng\ L^{-1}$ measured by SPME and $23 \pm 3\ ng\ L^{-1}$ measured by LLE (Figure 2 and Supplemental Data, Figure S7). Furthermore, the interlaboratory comparison of $C_{w,t}$ measured by SPME proves that the method is highly reproducible, even after transport and storage of samples. The time-weighted average $C_{w,t}$ values measured by laboratory 2 were equal to time-weighted average $C_{w,t}$ values measured by laboratory 1 for both HCB and OTP ($p = 0.822$ and 0.841, respectively; Supplemental Data, Figures S8 and S9). The excellent agreement of results from SPME and LLE allows to compare existing datasets obtained by conventional solvent extraction procedures with new results obtained by this alternative method in future experiments. Thus, one of the most important regulatory requirements for the replacement of routine methods is fulfilled.

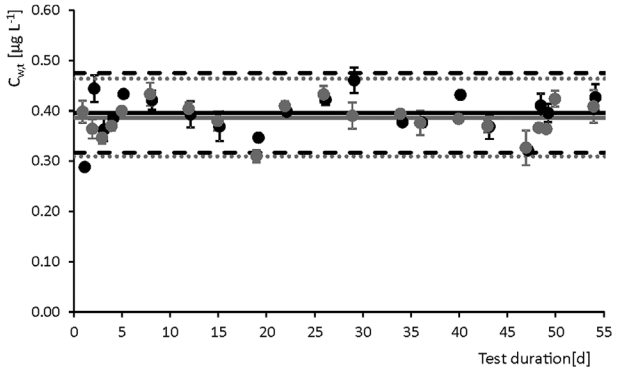

Figure 1. Total water concentrations ($C_{w,t}$) of hexachlorobenzene measured by solid-phase microextraction (black circles) in comparison with liquid–liquid extraction (gray circles; $n = 2$–4). Continous lines indicate time-weighted average concentrations ± 20% (dashed lines), and error bars show standard deviation.

$C_{w,f}$ derived from SPME analysis

For both HCB and OTP, no significant differences between $C_{w,t}$ and $C_{w,f}$ were obtained in the first study. Diligent removal of feces and uneaten food led to very low TOC concentrations of 1.2 to $1.8\ mg\ L^{-1}$ in the test vessel throughout the study, which is even below the maximum value tolerated by OECD guideline 305 for dilution water ($2\ mg\ L^{-1}$ TOC). Concentrations of TOC that are $<2\ mg\ L^{-1}$ can still significantly reduce $C_{w,f}$ of highly HOCs in batch sorption studies under equilibrium conditions [18]. However, this effect should be reduced by flow-through conditions as applied in this test system, which circumvents long-term equilibration of test substances with organic matter in the test system. Nevertheless, this still cannot explain that no differences occurred between $C_{w,t}$ and $C_{w,f}$ in the present study because the time between sampling and measurement by automated SPME should have led to further equilibration of HOCs with organic matter in the water phase of the closed vials. In this context, the occurrence of different organic matter fractions with differing potential to sorb HOCs has to be considered (compare the *Sorption of HOCs to organic matter* section). In the second study, time-weighted average $C_{w,f}$ values of PCB 153 were significantly reduced to $14\ ng\ L^{-1}$ ($p = 0.001$), which is a reduction of approximately 40% compared with the $C_{w,t}$ of $23\ ng\ L^{-1}$ (Figure 2). Potential uncertainties regarding the amount of $C_{w,f}$ are discussed in the following sections. Because of the higher hydrophobicity of PCB 153 compared with HCB and OTP, a higher sorption was expected. In addition, the TOC concentration was higher in the second study. Concentrations of 4 to $7\ mg\ L^{-1}$ TOC resulted in a time-weighted average concentration of $5.98\ mg\ L^{-1}$ TOC. The higher SD of $C_{w,f}$ compared with $C_{w,t}$ results from the fact that variations of the instrumental analysis (GC–MS) and varying fiber sensitivity (resulting from repeated usage of the fiber) cannot be corrected by internal standards.

Sorption of HOCs to organic matter

The organic matter fractions most relevant for sorption of HOCs in fish bioconcentration studies are fish feed and fish feces, which can occur both as particulate and dissolved organic matter. A previous investigation of organic matter relevant for bioconcentration studies revealed a very high sorption of HOCs

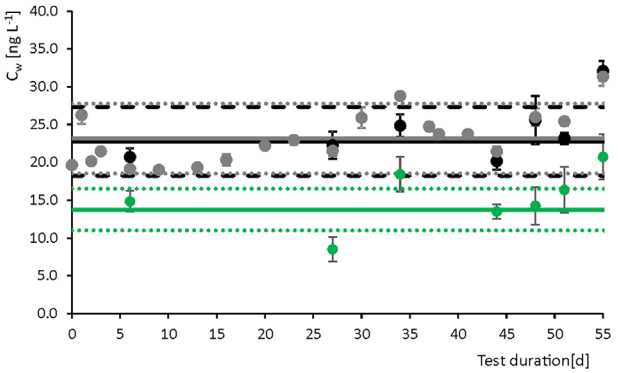

Figure 2. Water concentrations (C_w) of polychlorinated biphenyl 153 during the bioconcentration study. Gray circles: total water concentrations ($C_{w,t}$) measured by liquid–liquid extraction; black circles: $C_{w,t}$ measured by solid-phase microextraction (SPME); green circles: freely dissolved water concentrations ($C_{w,f}$) measured by SPME ($n = 4$–8). Continous lines indicate time-weighted average concentrations ± 20% (dashed lines), and error bars show standard deviation.

to organic matter from fish feed and the filter residue sampled from the outlet pipe of a control vessel [18]. These fractions are the potential causes for the reduction of $C_{w,f}$ compared with $C_{w,t}$ in the present study. In the first bioconcentration study with HCB and OTP, a time-weighted average concentration $<$ 2 mg L^{-1} TOC with a DOC content higher than the content of POC was measured (Supplemental Data, Figure S2). In contrast, in the second bioconcentration study with PCB 153, a time-weighted average concentration of 5.98 mg L^{-1} TOC in the test vessel was measured, with the content of POC predominating over the content of DOC (Supplemental Data, Figure S3). Depending on their composition, organic matter fractions have a different potential to sorb HOCs. As well as the very low TOC content in the first bioconcentration study, a different composition of organic matter compared with the previous batch sorption experiments [18] is a potential explanation for the fact that no measurable differences in $C_{w,t}$ and $C_{w,f}$ could be determined for HCB and OTP. A high content of filter residue-DOC and a low content of fish feed-DOC in the test vessels could be of special significance. Unlike fish feed-DOC, which was the most important constituent regarding the sorption of HOCs in previous sorption experiments [18], the sorption to DOC from filter residue was relatively low and could not be determined accurately. Roughly calculated, the logarithmic DOC–water partitioning coefficient ($\log K_{DOC}$) for HCB in filter residue-DOC was only approximately 4.0 compared with logarithmic TOC–water partitioning coefficients ($\log K_{TOC}$) and $\log K_{DOC}$ values of 4.95 to 5.38 for fish feed-TOC, filter residue-TOC, and fish feed-DOC. The fish feed pellets were observed to be mostly stable during feeding and until siphoning off of food residues and feces. This was assumed to have prevented the release of highly sorptive DOC from the fish feed pellets. However, based on the previously measured K_{TOC} values for PCB 153 ($\log K_{TOC}$ 5.96–6.01) [18], the difference between $C_{w,t}$ and $C_{w,f}$ in the second study cannot be explained by the quantitative occurrence of TOC in the test system. Based on the estimated time-weighted average concentration of 5.98 mg L^{-1} TOC and the reduction of $C_{w,f}$ compared with $C_{w,t}$, a K_{TOC} value of 109 500 ($\log K_{TOC}$ 5.04) can be calculated for PCB 153, which is approximately 10 times lower compared with the previous batch experiments. A further determination of sorption of PCB 153 to filter residue-DOC according to Böhm et al. [18] resulted in a K_{DOC} of 99 500 ($\log K_{DOC}$ 5.0; Supplemental Data, Figure S10), which is in the same range of sorption in the test media of the present study. However, because the content of POC was higher than the content of DOC in the second study (Supplemental Data, Figure S3), further parameters must be responsible to explain the discrepancies. Again, a difference in the composition of organic matter fractions is considered to be relevant, in particular regarding the composition of filter residue, or rather the specific organic matter in the test vessels. Because organic matter originates from fish feces, differing amounts of feed residues, and other constituents (e.g., bacteria), its composition is assumed to vary greatly between different fish studies. Furthermore, the natural occurring TOC in the present study was most likely different from the preparation of filter residue for the batch sorption studies, which was dried at 105 °C after a filtering process that had an influence on the organic matter composition. This assumption is supported by subsequently performed sorption experiments with PCB 153 and unmodified waters from a vessel with rainbow trout. Samples with a mean organic matter content of 3.12 mg L^{-1} TOC (range, 2.31–5.20 mg L^{-1}) resulted in a mean K_{TOC} value of 43 000, or rather a log K_{TOC} of 4.63 (K_{TOC} 12 500–112 000, log K_{TOC} 4.10–5.05), which matches the K_{TOC} value of 109 500 calculated from the second fish study and confirms the high variability of organic matter composition.

Consequences for BCF values

Because SPME and LLE led to equal $C_{w,t}$ for the 3 HOCs, no relevant differences have to be considered for the estimation of BCF values based on $C_{w,t}$. This is different if $C_{w,f}$ is taken into account. A potential effect for the first study can be derived from the K values. If an agreement of the batch sorption K_{DOC} value (filter residue-DOC) and the K_{TOC} value obtained from the fish study (as observed for the second study) is assumed as well for the first study, the effect of TOC on $C_{w,f}$ would have only led to a deviation of the BCF value for HCB of $<$2% (Equations 2 and 3). This demonstrated that an artificial influence of organic matter on BCF values can be reduced or even prevented by the efficient removal of organic matter from the test system, at least for HOCs in the range of log K_{OW} 5 to 6. In contrast, a significant impact on the BCF value was determined for the more hydrophobic PCB 153 at higher TOC concentrations. The BCF value calculated based on $C_{w,f}$ is 28 058 compared with 17 079 based on the calculation from $C_{w,t}$, which is an increase of approximately 65%. Although PCB 153 is much more hydrophobic than HCB ($\log K_{OW}$ 7.75 and 5.73, respectively), its BCF is lower (log BCF 4.23 and 4.47, respectively [5.36 and 5.70, respectively, on lipid weight basis]), which is in agreement with the leveling off of BCF values above log K_{OW} 5 to 6. If $C_{w,f}$ is taken into account, log BCF of PCB 153 increases to 4.45 (5.58 on lipid weight basis) and approaches the BCF of HCB. However, the results of the present study cannot explain the discrepancy of a missing BCF–K_{OW} correlation above log K_{OW} 5 to 6 by a reduction of $C_{w,f}$ that is caused by the presence of organic matter in the test system. The increase of the BCF for PCB 153 (if calculated based on $C_{w,f}$ instead of $C_{w,t}$) should have been orders of magnitude higher if the leveling off of BCF values could be solely explained by artificial influences of TOC in the present study. However, absolute conclusions on the amount of $C_{w,f}$ and on the discrepancy between BCF values calculated from $C_{w,t}$ and $C_{w,f}$ cannot be specified because of the potential of both the overestimation and underestimation of BCF values through method-inherent reasons. Generally, biotransformation of test substances in fish has to be considered as an influencing factor that could occur under laboratory conditions and would lead to a reduction of BCF values. However, according to Buckman et al. [40], one should not expect a significant biotransformation for PCB 153. The BCF values can be underestimated by an overestimation of $C_{w,f}$, which could occur when organic matter-bound molecules are desorbed to form a new equilibrium after significant depletion of the test substance by the SPME fiber [31,32] and by an accelerated transport of analytes to the fiber, which can occur through dissolved organic matter if the sampling time is insufficient [18,32,41–44]. Because sorption decreases with increasing temperature for many substances [45], BCF values could be underestimated when the extraction of $C_{w,f}$ is performed at a higher temperature than the temperature in the flow-through fish study. Because the lowest stable temperature that can be adjusted in the autosampler heating device is 30 °C, a difference in the water temperature in the fish study (approximately 15 °C) was technically unavoidable. In contrast, BCF values can be overestimated by an underestimation of $C_{w,f}$ with the present approach because nonequilibrium conditions of the flow-through study are not fully taken into account. (The time between sampling and measurement by automated SPME

leads to further equilibration in the vial and thus to additional sorption and reduced extraction.) Furthermore, the interaction of uptake pathways has to be considered as a likely influencing factor. Sorption of the test substances to fish feed before consumption by fish could imply a relevant impact of biomagnification on the evaluation of BCFs and would then result in an overestimation of bioconcentration. A batch experiment with PCB 153 and fish feed revealed immediate sorption after spiking and resulted in K_{TOC} values of 400 000 to 600 000 (log K_{TOC} 5.6–5.8) within 30 to 60 min. Details of the experiments and calculations are given in the Supplemental Data (see *Immediate Sorption* section). However, the impact of biomagnification on the present study is assumed to be of minor relevance because fish were fed with a very small feed ration of only 1.5% of body weight per day, which led to a very fast consumption of fish feed (<15 min). To explain the leveling off of BCF values solely by the organic matter present, aiming at a 1 to 1 relation of BCF on lipid weight basis and K_{OW}, the $C_{w,f}$ would need to be reduced by a factor of approximately 250 or rather by 2 orders of magnitude when compared with $C_{w,t}$. This is not likely to have occurred because of any of the influencing factors named previously. Instead, other reasons are assumed to be relevant for the lower than expected BCF of PCB 153, such as restricted membrane permeation of HOCs as a result of their molecular size [8,9,12]. This is as well supported by a significantly smaller (approximately 70%) uptake rate constant k_1 for PCB 153 compared with HCB and OTP (Supplemental Data, Table S7).

Applicability of SPME in BCF studies

Based on the results from the present study, the use of automated SPME for water analysis in fish bioconcentration studies can be recommended with some minor limitations. The results for the investigated substances showed that SPME can easily replace LLE for the measurement of $C_{w,t}$, guaranteeing a high quality of results. During method modification, the parameters extraction temperature, fiber coating, coating thickness, and extraction time have to be considered. While a remeasurement of extracts is possible after LLE, and storage of extracts requires minimum space, remeasurement of water samples after SPME is not possible (because of the depletion of analytes). Therefore, retained samples for SPME have to be original water samples, and additional freezer storage has to be considered. In contrast, by applying automated SPME, the use of organic solvents can be avoided and labor time can be saved. Most importantly, SPME allows the simultaneous determination of $C_{w,f}$ in addition to $C_{w,t}$, which would provide valuable information on the sorption of HOCs in the test system. Potential constraints can be uncertainties by both the potential over- and underestimation of $C_{w,f}$: because of depletion and accelerated transport (overestimation: the supposedly true $C_{w,f}$ should be lower than measured) or rather nonequilibrium conditions in the test versus equilibrium conditions during extraction (underestimation: the supposedly true $C_{w,f}$ should be higher than measured). Depletion and accelerated transport can be adversely influenced by time. If extraction time is increased, the effect of an accelerated transport is reduced but depletion is increased and vice versa. Both effects can be significantly reduced if SPME fibers with a reduced coating volume are used, for example 7 μm PDMS instead of 100 μm PDMS fibers. The effect of an accelerated transport of analytes toward the fiber can be further reduced by the enhancement of kinetic processes through an increase of agitation speed in the heating device of the autosampler. Because possible interferences could be resolved by passive sampling [27,46], equilibrium passive sampling could be applied to further confirm the reliability of nonequilibrium sampling by automated SPME. To consider as well the influence of flow-through conditions, SPME should be used in situ under equilibrium conditions, that is, fiber pieces are introduced into and equilibrated in the test vessels. Because of the large water volume in the test vessels and the continuous supply of test substances, possible interference by depletion could also be prevented. Furthermore, a temperature discrepancy between the fish study and extraction in the laboratory, which might influence the amount of extracted $C_{w,f}$ of test substances, could be avoided as well. In simple terms, while automated SPME under nonequilibrium conditions is less laborious and simplifies the extraction procedure, the use of in situ SPME would increase the precision of $C_{w,f}$. In this context, the disadvantages of in situ SPME are related to the procedure of handling and quantification. Fiber pieces equilibrated in situ have to be recollected and extracted with solvents unless additional equipment is used for the direct thermodesorption in the injector of a GC [27]. In either case, fiber length and coating volume of the fibers have to be known for quantification, and external calibration is commonly not performed with fibers but by the injection of solvent-based stock solutions. Unlike the automated SPME approach, $C_{w,f}$ is not directly derived from the external calibration but is calculated from the concentration of the test substance in the fiber coating and specific fiber–water partition coefficients, which provide information about the distribution of the test substance between the water phase and the fiber coating under equilibrium conditions. This would be a highly relevant issue in case fiber–water partition coefficients are lacking, especially in the regulatory context when new chemicals are tested for a potential authorization. Quantification from extraction under nonequilibrium conditions is even more laborious compared with automated SPME, because $C_{w,f}$ values in the test vessels change frequently, but equilibration between test substances and fiber can last up to several days. Continuous adaption to altering concentrations because of changes in the supply of test substances or changes in the amount of organic matter in the test system further delay the equilibration process. Therefore, the measurement of specific $C_{w,f}$ values at defined sampling intervals is hampered, unless the fibers are used under nonequilibrium conditions with stable isotope calibration [47]. Otherwise, in situ SPME could allow for a time-integrated measurement of $C_{w,f}$. After sufficient validation in OECD guideline 305 test systems, the use of in situ SPME could be advantageous in addition to automated SPME to validate $C_{w,f}$. However, because of the comparatively simple handling in the laboratory, automated SPME is recommended in any case as the main method for the measurement of $C_{w,t}$. As a benefit, $C_{w,f}$ is determined simultaneously without further effort. In case of uncertainty regarding potential interfering factors, $C_{w,f}$ could at least be used in terms of a screening tool to elucidate the impact of organic matter on $C_{w,f}$ of highly HOCs, because it will most probably be more precise compared with a calculation of the possible organic matter impact from literature data.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3854.

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The results from this project provoked the recommendation of SPME for water analysis of HOCs in fish bioconcentration studies within the revised OECD guideline 305. The results of the present study will be integrated into an upcoming Guidance Document for the revised OECD guideline 305 [39].

Data Availability—Essential data are presented in the manuscript. Raw data are available on request from the corresponding author (leonard.boehm@umwelt.uni-giessen.de).

REFERENCES

1. Organisation for Economic Co-operation and Development. 2012. Test No. 305: Bioaccumulation in fish: Aqueous and dietary exposure. *OECD Guidelines for the Testing of Chemicals*. Paris, France.
2. Barcelo D. 1993. Environmental Protection Agency and other methods for the determination of priority pesticides and their transformation products in water. *J Chromatogr A* 643:117–143.
3. Mahara BM, Borossay J, Torkos K. 1998. Liquid-liquid extraction for sample preparation prior to gas chromatography and gas chromatography-mass spectrometry determination of herbicide and pesticide compounds. *Microchem J* 58:31–38.
4. Robles-Molina J, Gilbert-López B, García-Reyes JF, Molina-Díaz A. 2013. Comparative evaluation of liquid-liquid extraction, solid-phase extraction and solid-phase microextraction for the gas chromatography-mass spectrometry determination of multiclass priority organic contaminants in wastewater. *Talanta* 117:382–391.
5. Arias AH, Souissi A, Roussin M, Ouddane B, Souissi S. 2016. Bioaccumulation of PAHs in marine zooplankton: An experimental study in the copepod *Pseudodiaptomus marinus*. *Environ Earth Sci* 75:691.
6. Anastas PT, Warner JC. 1998. *Green Chemistry: Theory and Practice*. Oxford University, Oxford, UK.
7. Sheldon RA. 2016. Green chemistry and resource efficiency: Towards a green economy. *Green Chem* 18:3180–3183.
8. Opperhuizen A, van der Velde EW, Gobas FAPC, Liem DAK, van der Steen JMD, Hutzinger O. 1985. Relationship between bioconcentration in fish and steric factors of hydrophobic chemicals. *Chemosphere* 14:1871–1896.
9. Gobas FAPC, Lahittete JM, Garofalo G, Shiu WY, Mackay D. 1988. A novel method for measuring membrane-water partition coefficients of hydrophobic organic chemicals: Comparison with 1-octanol-water partitioning. *J Pharm Sci* 77:265−272.
10. Gobas FAPC, Clark KE, Shiu WY, Mackay D. 1989. Bioconcentration of polybrominated benzenes and biphenyls and related superhydrophobic chemicals in fish—Role of bioavailability and elimination into the feces. *Environ Toxicol Chem* 8:231−245.
11. Chessells M, Hawker CW, Connell DW. 1992. Influence of solubility in lipid on bioconcentration of hydrophobic compounds. *Ecotoxicol Environ Saf* 23:260–273.
12. Dimitrov SD, Dimitrova NC, Walker JD, Veith GD, Mekenyan OG. 2002. Predicting bioconcentration factors of highly hydrophobic chemicals. Effects of molecular size. *Pure Appl Chem* 74:1823–1830.
13. Jonker MTO, van der Heijden SA. 2007. Bioconcentration factor hydrophobicity cutoff: An artificial phenomenon reconstructed. *Environ Sci Technol* 41:7363–7369.
14. Nendza M, Müller M. 2010. Screening for low aquatic bioaccumulation (1): Lipinski's 'Rule of 5' and molecular size. *SAR QSAR Environ Res* 21:495–512.
15. Muir DCG, Hobden BR, Servos MR. 1994. Bioconcentration of pyrethroid insecticides and DDT by rainbow trout: Uptake, depuration, and effect of dissolved organic carbon. *Aquat Toxicol* 29:223–240.
16. Ramos EU, Meijer SN, Vaes WHJ, Verhaar HJM, Hermens JLM. 1998. Using solid-phase microextraction to determine partition coefficients to humic acids and bioavailable concentrations of hydrophobic chemicals. *Environ Sci Technol* 32:3430–3435.
17. Haitzer M, Höss S, Traunspurger W, Steinberg C. 1998. Effects of dissolved organic matter (DOM) on the bioconcentration of organic chemicals in aquatic organisms—A review-. *Chemosphere* 37: 1335–1362.
18. Böhm L, Schlechtriem C, Düring R-A. 2016. Sorption of highly hydrophobic organic chemicals to organic matter relevant for fish bioconcentration studies. *Environ Sci Technol* 50:8316–8323.
19. Arnot JA, Gobas FAPC. 2006. A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. *Environ Rev* 14:257–297.
20. Arthur CL, Pawliszyn J. 1990. Solid phase microextraction with thermal desorption using fused silica optical fibers. *Anal Chem* 62:2145–2148.
21. Risticevic S, Lord H, Górecki T, Arthur CL, Pawliszyn J. 2010. Protocol for solid-phase microextraction method development. *Nat Protoc* 5:122–139.
22. Düring R-A, Böhm L, Schlechtriem C. 2012. Solid-phase microextraction for bioconcentration studies according to OECD TG 305. *Environ Sci Eur* 24:4.
23. Spietelun A, Kloskowski A, Chrzanowski W, Namieśnik J. 2013. Understanding solid-phase microextraction: Key factors influencing the extraction process and trends in improving the technique. *Chem Rev* 113:1667–1685.
24. Bridle HL, Heringa MB, Schäfer AI. 2016. Solid-phase microextraction to determine micropollutant-macromolecule partition coefficients. *Nat Protoc* 11:1328–1344.
25. Piri-Moghadam H, Ahmadi F, Pawliszyn J. 2016. A critical review of solid phase microextraction for analysis of water samples. *Trends Anal Chem* 85:133–143.
26. Fischer FC, Böhm L, Höss S, Möhlenkamp C, Claus E, Düring R-A, Schäfer S. 2016. Passive dosing in chronic toxicity tests with the nematode *Caenorhabditis elegans*. *Environ Sci Technol* 50:9708–9716.
27. Witt G, Lang SC, Ullmann D, Schaffrath G, Schulz-Bull D, Mayer P. 2013. Passive equilibrium sampler for in situ measurements of freely dissolved concentrations of hydrophobic organic chemicals in sediments. *Environ Sci Technol* 47:7830–7839.
28. Mayer P, Vaes WH, Hermens JLM. 2000. Absorption of hydrophobic compounds into the poly(dimethylsiloxane) coating of solid-phase microextraction fibers: High partition coefficients and fluorescence microscopy images. *Anal Chem* 72:459–464.
29. Smedes F, Geertsma RW, Zande TVD, Booij K. 2009. Polymer−water partition coefficients of hydrophobic compounds for passive sampling: Application of cosolvent models for validation. *Environ Sci Technol* 43:7047–7054.
30. Gilbert D, Witt G, Smedes F, Mayer P. 2016. Polymers as reference partitioning phase: Polymer calibration for an analytically operational approach to quantify multimedia phase partitioning. *Anal Chem* 88:5818–5826.
31. Zeng EY, Noblet JA. 2002. Theoretical considerations on the use of solid-phase microextraction with complex environmental samples. *Environ Sci Technol* 36:3385–3392.
32. Heringa MB, Hermens JLM. 2003. Measurement of free concentrations using negligible depletion-solid phase microextraction (nd-SPME). *Trends Anal Chem* 22:575–587.
33. Rodriguez-Lafuente A, Piri-Moghadam H, Lord HL, Obal T, Pawliszyn J. 2016. Inter-laboratory validation of automated SPME-GC/MS for determination of pesticides in surface and ground water samples: Sensitive and green alternative to liquid-liquid extraction. *Water Qual Res J Can* 51:331–343.
34. Pawliszyn J. Ed. 2011. *Handbook of Solid Phase Microextraction*. Elsevier, Waltham, MA, USA.
35. Bondarenko S, Gan J. 2009. Simultaneous measurement of free and total concentrations of hydrophobic compounds. *Environ Sci Technol* 43:3772–3777.
36. Vaes WHJ, Ramos EU, Verhaar HJM, Seinen W, Hermens JLM. 1996. Measurement of the free concentration using solid-phase microextraction: Binding to protein. *Anal Chem* 68:4463–4467.
37. Poerschmann J, Zhang Z, Kopinke F-D, Pawliszyn J. 1997. Solid phase microextraction for determining the distribution of chemicals in aqueous matrices. *Anal Chem* 69:597–600.
38. Schlechtriem C, Böhm L, Bebon R, Bruckert H-J, Düring R-A. 2017. Fish bioconcentration studies with column generated analyte concentrations of highly hydrophobic organic chemicals. *Environ Toxicol Chem* 36:906–916.
39. Organisation for Economic Co-operation and Development. 2016. Draft Guidance Document for Aspects of OECD TG 305 on Fish Bioaccumulation. [cited 2017 May 7]. Available from: http://www.oecd.org/chemicalsafety/testing/12%20December%202016_OECD%20305%20GD%20revised_clean.pdf
40. Buckman AH, Wong CS, Chow EA, Brown SB, Solomon KR, Fisk AT. 2006. Biotransformation of polychlorinated biphenyls (PCBs) and bioformation of hydroxylated PCBs in fish. *Aquat Toxicol* 78:176–185.

41. Kopinke F-D, Georgi A, Mackenzie K. 2001. Sorption and chemical reactions of PAHs with dissolved humic substances and related model polymers. *Acta Hydrochim Hydrobiol* 28:385–399.
42. Mayer P, Fernqvist MM, Christensen PS, Karlson U, Trapp S. 2007. Enhanced diffusion of polycyclic aromatic hydrocarbons in artificial and natural aqueous solutions. *Environ Sci Technol* 41:6148–615.
43. Haftka JJH, Parsons JR, Govers HAJ, Ortega-Calvo J-J. 2008. Enhanced kinetics of solid-phase microextraction and biodegradation of polycyclic aromatic hydrocarbons in the presence of dissolved organic matter. *Environ Toxicol Chem* 27:1526–1532.
44. ter Laak TL, van Eijkeren JCH, Busser FJM, van Leeuwen HP, Hermens JLM. 2009. Facilitated transport of polychlorinated biphenyls and polybrominated diphenyl ethers by dissolved organic matter. *Environ Sci Technol* 43:1379–1385.
45. Delle Site A. 2001. Factors affecting sorption of organic compounds in natural sorbent/water systems and sorption coefficients for selected pollutants. A review. *J Phys Chem Ref Data* 30:187–439.
46. Adolfsson-Erici M, Åkerman G, McLachlan MS. 2012. Measuring bioconcentration factors in fish using exposure to multiple chemicals and internal benchmarking to correct for growth dilution. *Environ Toxicol Chem* 31:1853–1860.
47. Cui X, Bao L, Gan J. 2013. Solid-phase microextraction (SPME) with stable isotope calibration for measuring bioavailability of hydrophobic organic contaminants. *Environ Sci Technol* 47:9833–9840.