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Using Solid-Phase Microextraction To Determine Partition Coefficients to Humic Acids and Bioavailable Concentrations of Hydrophobic Chemicals

EÑAUT URRESTARAZU RAMOS, SANDRA N. MEIJER, WOUTER H. J. VAES, HENK J. M. VERHAAR, AND JOOP L. M. HERMENS*

Research Institute of Toxicology (RITOX), Environmental Toxicology and Chemistry Section, Utrecht University, P.O. Box 80.176, NL-3508 TD Utrecht, The Netherlands

In the current study, the suitability of negligible depletion solid-phase microextraction (nd-SPME) to determine free fractions of chemicals in aquatic environments was explored. The potential interferences of the dissolved matrix (i.e., humic acids) with the SPME measurements were tested. Results show that nd-SPME measures only the freely dissolved fraction and that the measurements are not disturbed by the humic acids. In addition, nd-SPME was used to determine partition coefficients between dissolved organic carbon and water for four hydrophobic chemicals. Obtained values are in excellent agreement with previously reported data. Finally, the bioaccumulation of hexachlorobenzene and PCB 77 to *Daphnia magna* was determined in the presence and absence of humic acids. The bioconcentration factors (BCF) were calculated based on total as well as on free concentration. Lower BCF values are obtained in the presence of humic acids using total concentrations, whereas equal BCFs are found using free concentrations measured with nd-SPME. Therefore, we can conclude that negligible depletion SPME is a good technique to determine bioavailable concentrations of hydrophobic chemicals in aquatic environments.

Introduction

Concentrations of dissolved organic carbon (DOC) in natural waters range from 0.5 to 50 mg/L. DOC consists for 50−75% of humic substances, which result from the decomposition of dead material (*1*). A decrease in the freely dissolved aqueous concentration of organic pollutants has been found in the presence of DOC (*2*−*5*). Since it is generally accepted that only the freely dissolved fraction is available for uptake by organisms (*6*), this decrease is responsible for a reduced bioaccumulation or toxicity of chemicals to organisms when DOC is present (*7*−*13*).

Determining the free concentration of pollutants in aqueous samples containing DOC is problematic, since DOC is essentially inseparable from the aqueous phase. Standard liquid (or solid) phase extractions tend to maximize the extraction efficiency, reflecting total rather than free concentration. Methods to determine free concentrations in the presence of DOC and/or DOC partition coefficients have been published. Dialysis membranes as well as separation with solid-phase cartridges are used to separate dissolved from bound or associated fractions (*14*). The binding capacity of very low soluble pollutants toward humic acids was assessed by Chiou et al. (*15*) using solubility enhancement, while Resendes et al. (*4*) measured headspace concentration of aqueous solutions. For fluorescent pollutants, the binding capacity can be monitored by fluorescent quenching (*16*). Södergen et al. (*17*) as well as Huckins et al. (*18*) described the use of artificial lipophilic material to estimate bioaccumulation and bioavailability of hydrophobic chemicals in natural waters. In our laboratory, the solid-phase extraction disk (Empore disk) has been applied to measure freely dissolved concentrations (*11, 19*−*21*). For semivolatiles, equilibration through the headspace has been applied in the presence of biomacromolecules as the dissolved matrix (*22*).

However, some of these methods (e.g., fluorescence quenching, solubility enhancement, headspace equilibration) are restricted to a certain type of chemical while others (e.g., dialysis membranes, headspace equilibration, semipermeable membrane devices, Empore disks) involve long equilibration/sampling times, and many of these techniques are laborious and involve many manipulation steps. In addition, few of these methods are suitable to determine the free concentration in environmental samples, because they involve a significant depletion of the analyte (i.e., equilibria between the freely dissolved and the matrix-associated chemicals are disturbed).

Solid-phase microextraction (SPME), developed by Arthur and Pawliszyn (*23*), presents a very promising method to determine true free concentrations of organic chemicals. An SPME device consists of a small fused silica fiber coated with a polymeric stationary phase. The fiber is placed in a water sample, and chemicals partition into the coating, which can subsequently be thermally desorbed in a GC injector. SPME analyses can be performed in such a way (using a large sample/fiber volume ratio) that the extraction does not lead to any significant depletion of the sample. In addition, SPME sampling is quick, and extraction and analysis are performed in a single step.

Kopinke et al. (*24*) implicitly assumed that, since only the freely dissolved chemical partitions to the SPME fiber, SPME is suitable for measuring free fractions of dissolved compounds. Vaes et al. (*25*) independently proved this to be the case, by using negligible depletion SPME (nd-SPME) to determine free concentrations in systems containing proteins. Later nd-SPME was applied in several in vitro systems and also to determine membrane/water partition coefficients of several organic chemicals (*26*). Vaes et al. (*25*) stated that in order to measure free concentrations using SPME two requirements had to be fulfilled. (a) The amount of chemical extracted from the sample has to be minimized in order to not disturb the equilibria between the chemical and the matrix. They set the maximum extraction efficiency on 5%. (b) In addition, the matrix present in the sample should not disturb the kinetics of the absorption of the chemical into the SPME fiber. So that the measurements (which were made in the kinetic phase of the absorption) were not different from the calibration runs.

Recently, Poerschmann et al. (*27, 28*) have applied nd-SPME for determining partition coefficients between water and dissolved organic matter for highly hydrophobic chemicals. In these experiments the maximum extractable fraction

* Corresponding author phone: +31-30-253 54 00; fax: +31-30-253 50 77; e-mail: j.hermens@ritox.vet.uu.nl.

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was set at 10% of the total amount. Beside, the measurements were conducted at equilibrium sampling conditions, so that the measurements were not affected by potential influence of the matrix on the absorption kinetics.

The aim of the present study is to investigate the possibility of applying negligible depletion SPME to measure the free concentrations of highly hydrophobic chemicals in aquatic samples containing humic acids (HA). To validate the method, potential interferences posed by the presence of humic acids are investigated separately. For this purpose, the measurements of two samples (with and without humic acids) with identical free concentration are compared. In addition, the technique is used to determine the DOC/water partition coefficients. K_{DOC} values are determined by measuring the decrease in the free fraction of the chemical when adding different amounts of humic acids. Finally, the method is validated by studying the effects of humic acids on bioaccumulation of chemicals in *Daphnia magna*. The purpose is to investigate if the reduction in bioaccumulation, when humic acids are present, can be quantified by measuring the free concentration, as determined using nd-SPME.

Experimental Section

Materials. The 1 cm long SPME fibers coated with poly(dimethylsiloxane) (7 μm thickness) were purchased from Supelco (Bellefonte, CA). New fibers were conditioned in a GC injector at 300 °C for 2 h; in addition, the fibers were conditioned daily at 275 °C for 10 min. The chemicals were of >99% purity: pentachlorobenzene (PeClBz), hexachlorobenzene (HxClBz), and 4,4′-dichlorodiphenyltrichloroethane (DDT) were purchased from Riedel de-Haën AG (Seelze, Germany), and 3,3′,4,4′-tetrachlorobiphenyl (PCB 77) was purchased from Labor Ehrenstorfer (Augsburg, Germany). Humic acid sodium salt was obtained from Aldrich-Chemie (Steinheim, Germany). The organic carbon content of the humic acids (34%) was determined at the WRK Laboratory (Nieuwegein, The Netherlands) using a total organic carbon analyzer (model 700, OI Analytical, College Station, TX). A colorimetry kit for determination of total lipids was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Aqueous solutions of the chemicals were prepared in buffered water (0.1 M phosphate) using the generator disk in the headspace method as described by Urrestarazu Ramos et al. (*29*).

SPME Analyses. Two different SPME sampling systems were used in this study. (a) Manual sampling was performed in 275-mL round bottles stirred with a magnetic stirrer at 1000 rpm. The 1 cm long SPME fibers were used in this system, and GC analyses were conducted using a Carlo Erba Megaseries 5300 gas chromatograph (Carlo Erba, Milano, Italy). (b) Automated sampling was performed using either 2 or 15 mL vials placed on a Varian star 3600 cx GC equipped with a Varian 8200 cx SPME autosampler (Varian, Palo Alto, CA). This autosampler device incorporates an agitation system for SPME sampling. For this system, fibers were cut to a 1 mm length.

The GCs were equipped with 15 m J&W Scientific DB 5.625 capillary columns with a film thickness of 0.25 μm and internal diameter of 0.32 mm. The SPME fiber was desorbed in the splitless injector at 275 °C for 5 min during which the column temperature was kept at 50 °C. The injector was then switched to the split mode, and subsequently the fiber was withdrawn and the column temperature increased at either 15 or 30 °C/min up to 280 °C (stationary for 10 min). An electron capture detector at 350−365 °C was used for detection.

Absorption Profiles to SPME Fibers. Negligible depletion SPME implies that the amount absorbed by the fiber is small as compared to the amount that is freely dissolved, so that the equilibria in the sample are not disturbed (*25*). Therefore, it is necessary to know the amount of depletion of the system, and this amount can be determined by measuring the kinetics of the absorption process. In addition, since sampling does not necessarily have to be at equilibrium, knowledge of the absorption kinetics provides information for determining optimum sampling times (*25*). For this purpose, absorption profiles were determined for each compound. This procedure is explained elsewhere (*25*). In short, the fiber is exposed at varying exposure times to an aqueous solution containing the chemicals under study. Subsequently, the fiber is desorbed in a splitless injector of a GC. Since the volume of the fiber and the water and the aqueous concentration are known, the resulting time vs concentration data can be fitted to an appropriate kinetic model. [See Vaes et al. (*25*) for detailed information on the application of the one compartment kinetic model in SPME.] This model can be simplified when the depletion is negligible:

$$V_a/V_f \gg k_1/k_2 = K_{SPME} \quad (1)$$

where k_1 and k_2 are the uptake and elimination rate constants, respectively; V_a and V_f are the volume of water and fiber, respectively; and K_{SPME} is the partition coefficient between water and the SPME fiber. As mentioned above, this is a necessary condition for not disturbing the equilibria of the analyte with the sample.

The profiles were determined for the four chemicals under study. Phosphate-buffered Millipore water (pH 7.0) was contaminated with each chemical. The aqueous concentrations were determined using solvent extraction and analyzed on a GC as described above.

In the manual sampling procedure, the concentrations were 116, 87.3, 43.8, and 70.7 ng/L for PeClBz, HxClBz, PCB 77, and DDT, respectively, and the exposure times were 2, 5, 10, 15, 30, and 60 min. For the autosampler profiles, 2-mL vials containing 1.5 mL of solution were used. The aqueous concentrations were 3.81 and 3.84 μg/L for PeClBz and HxClBz, respectively. The exposure times were 1, 2, 4, 6, 8, 10, 15, 20, or 30 min. In both cases, each time point was measured in duplicate, and for every measurement a new sample was taken. Directly after exposure, the fiber content was analyzed on the GC as described above.

The volume of the polymer coating is 0.026 and 0.0026 μL for 1 cm and 1 mm fiber length, respectively. The manual sampling data were fitted to the one-compartment model assuming negligible depletion, which is allowed because of the high water/fiber volume ratio (275 mL:0.026 μL). On the other hand, depletion was expected for the autosampler system (1.5 mL:0.0026 μL); therefore, these data were fitted to the general one-compartment model (*25*). The curve fitting was performed using the Systat v. 5.0 statistical program (Systat Inc., Evanston, IL). Estimates of k_1 and k_2 were obtained, and K_{SPME} was calculated as k_1/k_2.

Interferences. It is assumed that only freely dissolved molecules partition to the SPME and that therefore only the free concentration is determined. However, the measurement might be influenced by the humic acids present in the water. It has been suggested (*30*) that humic acids can bind to the SPME coating, at long exposure times (30−60 min). This would then result in an overestimation of the free fraction because of the extra amount of chemical associated with the humic acids that are bound to the fiber. Another source of interferences can result from the presence of a static layer around the fiber (i.e., local depletion) if sampling at non-equilibrium conditions (*31*). In this case, the free fraction would also be overestimated if the diffusion in water of the bound chemical is faster than the diffusion of the free chemical.

To validate the use of negligible depletion SPME to determine freely dissolved concentrations of highly hydro-

phobic chemicals in the presence of humic acids, the measurements of two solutions with identical free concentration (one with humic acids and the other without) were compared. The solutions were prepared by equilibration through the gas phase as described by van Wezel et al. (*22*). The system consists of two round-bottomed flasks that are connected by a bent tube. Both flasks were filled with an aliquot of an aqueous solution of the compound under study, and a HA solution was added to one of these flasks. Then the two flasks were connected by the vapor-tight air bridge. Since only the unbound chemical diffuses through the vapor phase, the free concentration in both bottles will equilibrate; i.e., at equilibrium both solutions will behave as if they were a single solution, including HA. Therefore, a nondisturbed measurement on both compartments should yield indistinguishable results.

A solution of PCB 77 (±500 ng/L) and a solution of HA (180 mg/L) were prepared in phosphate-buffered sterile water (pH 8.0). One flask was filled up with 90 mL of PCB 77 solution, and the other was filled with 80 mL of PCB 77 solution and 10 mL of HA solution (final HA concentration was 20 mg/L). According to van Wezel et al. (*22*), the system should be in equilibrium after 2 weeks. After this period of time, five 12-mL samples were taken from each flask and analyzed by SPME. Sampling time was 1 min using the autosampler system. The total concentrations were determined by extraction with 2 mL of hexane and GC analysis.

***K*$_{DOC}$ Determination.** Partition coefficients between dissolved organic carbon (DOC) and water were determined for all four chemicals. The decrease in the free concentration after the addition of HA was measured using SPME. Solutions in Millipore water (phosphate buffer, pH 7.0) were prepared for each chemical. HA stock solutions were prepared by dissolving humic acids in the buffered water and adjusting the pH to 7.0. Final solutions were obtained by mixing 360 mL of contaminated solution and 40 mL of HA stock solution and shaking to equilibrate for at least 45 min. Final humic acid concentrations were 0.1, 1, 10, 50, and 100 mg/L for PeClBz and HxClBz and 0.1, 1, 5, 10, and 25 mg/L for PCB 77 and DDT. For each HA concentration, duplicate samples were prepared and measured. Since the method assumes that the reduction in free concentration is only due to absorption to the HA, the total concentration of the samples was analyzed using liquid−liquid extraction. The measurements were carried out using manual sampling for 20 min, and GC analyses were performed as described above.

The free fraction at different humic acid concentrations can be fitted to the following expression (*32*):

$$f = \frac{1}{1 + K_{HA}C_{HA}} \qquad (2)$$

where f is the free fraction (defined as the ratio between the free and total concentrations), K_{HA} is the HA/water partition coefficient of the chemical, and C_{HA} is the aqueous concentration of humic acids. K_{HA} in eq 2 was fitted to experimental values using the Systat v. 5.0 program (Systat Inc., Evanston, IL). K_{DOC} was derived by normalizing K_{HA} to the carbon content of the humic acids.

Bioavailability to *Daphnia magna*. Bioconcentration factors (BCF) of HxClBz and PCB 77 to *Daphnia magna* in the presence and absence of humic acids were determined. Daphnids bred in-house were used as test organism. Dutch standard water (DSW) (*33*) was used as culture and test water. To avoid chemicals binding to particulate matter potentially present in this generated water, the water was filtered using either a 0.2 or a 0.45 μm filter. Humic acid stock solutions were prepared by dissolving humic acid sodium salt in DSW and adjusting the pH to that of DSW (± 8.0).

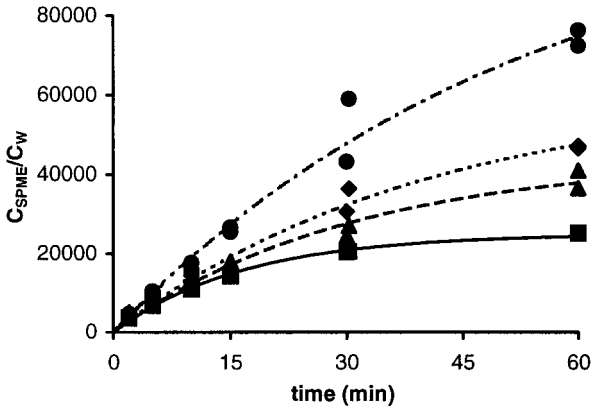

FIGURE 1. Absorption profiles obtained by manual sampling for the four chemicals under study. The lines represent the fitted one compartment model. (■ —, pentachlorobenzene; ▲ — —, hexachlorobenzene; ♦ - - -, PCB 77; ● — - —, DDT).

An 8-L test solution was prepared by dilution of both stocks (chemical and humic acids). Concentrations of HA after dilution were 108 mg/L for HxClBz and 7.8 mg/L for PCB 77. Concentrations of the chemicals after dilution were approximately 100 times lower than the predicted 48h-EC50 for baseline toxicity (*34*). The solutions were allowed to equilibrate for at least 1 h, before adding the daphnids to the aquaria. The aquaria were covered with glass plates. The pH (8.0 ± 0.2), temperature (20 ± 1 °C), and dissolved oxygen content (above 95%) were measured in each aquarium at the beginning and end of each experiment. A 12-h light/dark cycle was maintained during the experiment. Sixty daphnia were exposed for 2−3 days in each aquarium.

Aqueous concentrations in each aquarium were measured, in duplicate, at the beginning and end of the experiments. SPME was used to determine the free concentration and liquid−liquid extraction for total concentration. The SPME sampling was performed using 12-mL samples in the autosampler. The 1-min sampling was used for HxClBz, and 10 min was used for PCB 77. SPME calibration samples were prepared by dilution of the original stock. The actual concentration of the stock solution was determined by liquid−liquid extraction and GC analysis.

After exposure, the aquarium contents were filtered trough a sieve, and the retrieved daphnids were flushed with Millipore water. The daphnids were extracted as follows. Five groups of 10 individuals were pooled in Eppendorf cups containing 100-μL of Millipore water. Subsequently, 400 μL of hexane was added, and the daphnids were disintegrated by sonication for 20 s using an exponential microprobe of 3 mm at an amplitude of 8−10 μm (MSE Soniprep 150 Ultrasonic disintegrator, MSE Scientific Instruments, Sussex, England). The samples were then centrifuged at 13000 rpm for 1 min, and the hexane fraction was analyzed on the GC.

The lipid content of the daphnids was determined using a lipid content determination kit. In short, the samples were measured spectrophotometrically (530 nm) after reaction with sulfuric acid and color reagent (13 mmol/L vanillin in concentrated phosphoric acid). Bioconcentration factors on lipid content basis (BCF = C_l/C_a) were calculated based on total aqueous concentration (C_a measured with LLE) and free aqueous concentration (C_a using SPME).

Results and Discussion

Absorption Profiles to SPME Fibers. Absorption profiles, as shown in Figure 1, can be adequately described by a one-compartment kinetic model. The parameter estimates and statistics of the curve fitting of the manual absorption profiles are shown in Table 1. It is observed that for manual sampling

TABLE 1. Kinetic Constants of the Process of Absorption to the Fiber for the Two Systems Described in the Text

chemical	log K_{OW}[a]	$k_1 \pm$ SE (min^{-1})	$k_2 \pm$ SE (min^{-1})	log $K_{SPME} \pm$ SE	N[b]	depletion (%)
			Manual Sampling			
Pentachlorobenzene	5.18	1503 ± 75	0.0589 ± 0.0042	4.41 ± 0.06	12	0.2
Hexachlorobenzene	5.73	1450 ± 110	0.0328 ± 0.0047	4.65 ± 0.11	12	0.4
PCB 77	6.63	1530 ± 170	0.0254 ± 0.0044	4.78 ± 0.15	12	0.6
DDT	6.91	2090 ± 180	0.0188 ± 0.0043	5.05 ± 0.17	12	1.0
			Autosampler			
Pentachlorobenzene		8760 ± 540	0.2300 ± 0.0180	4.58 ± 0.07	18	6
Hexachlorobenzene		43400 ± 1500	0.2282 ± 0.0096	5.28 ± 0.03	18	25

[a] Octanol/water partition coefficients obtained from ClogP (Biobyte, Corp., Claremont, CA). [b] Number of data points used in the curve fitting procedure.

the uptake rate constant (k_1) remains practically constant, whereas the elimination rate constant (k_2) decreases with increasing the hydrophobicity. It has been discussed, by Vaes et al. (*35*) among others, that this is an indication of diffusion of compounds through the stagnant layer being the rate-limiting step of the absorption process. Therefore, in this case, absorption kinetics can be improved by optimizing the agitation of the system. This then implies that the hydrophobicity range at which kinetics are governed by diffusion through the polymeric phase can be widened. But at a certain hydrophobicity range, the stagnant layer will still be the rate-limiting step. The estimates of the curve fitting for profiles established with the autosampler are also shown in Table 1. Although data are available for only two of the chemicals, a trend is observed indicating that k_2 remains constant and k_1 increases with increasing hydrophobicity. Therefore, the autosampler agitation system reduces the stagnant layer to a minimum, and the kinetics cannot be further improved because they are limited by the diffusion through the polymeric phase.

As discussed above, the presence of a stagnant layer surrounding the SPME fiber can be problematic when measuring free concentrations (*31*). If measurements are done in the kinetic phase, the diffusion of the bound fraction may play an important role, in the case that diffusion of the bound chemical is faster than that of the free chemical. Therefore, care must be taken when determining the free concentrations during the uptake phase if the water-to-SPME kinetics are governed by diffusion trough the stagnant layer.

The estimated kinetic parameters were used to set the sampling conditions. Sampling volumes were selected in order to minimize the total depletion (<5%). The sampling times were selected as described by Vaes et al. (*25*). The following sampling conditions were used in further experiments: 275 mL of sample, 1 cm long fiber, and 20 min sampling for the manual system; a 1.5 mL of sample, 1 mm long fiber, and 1 min for the autosampler.

Table 1 also shows the determined K_{SPME} values in the two sampling systems. Most of the data points measured using the autosampler are virtually at steady state, whereas the data obtained by manual sampling are still relatively far from the steady-state situation. Therefore, K_{SPME} for the manual sampling are extrapolated and consequently less reliable. In addition, since the fibers were cut to 1 mm for the autosampler system, there exists a uncertainty in the volume of the polymeric phase.

Interferences. The next step was to validate negligible depletion SPME to determine free concentrations in the presence of humic acids. More specifically, we wanted to investigate possible interferences in the measurements by the humic acids. After equilibration through the headspace of two flasks (one containing HA), the total concentration in the flask containing HA was about 5 times higher (670 ng/L) than in the flask without HA (134 ng/L). Log K_{DOC} can be estimated from the free fraction (f) as the ratio between the total concentration in both flasks and eq 2. The value obtained by this procedure (5.75) is very close to the one determined using SPME (5.97, shown in Table 2). This is a good indication that the solutions were indeed at equilibrium, and therefore the free concentrations were equal in both flasks.

TABLE 2. DOC/Water Partition Coefficients Determined in This Study (Using eq 2) and Values Reported in Literature

	this study			literature	
chemical	log K_{DOC}	± SE	N[a]	log K_{DOC}	ref
pentachlorobenzene	4.50	± 0.03	14	4.6	*4*
hexachlorobenzene	4.97	± 0.05	14	5.1	*11*
PCB 77	5.97	± 0.12	10	not available	
DDT	5.57	± 0.18	10	5.1−6.0	*2, 3, 11,14*

[a] Number of data points used in the curve fitting procedure.

The nd-SPME measurements ($N = 5$) of both flasks resulted in area counts of 149 000 ± 21 000 and 154 000 ± 22 000 for the samples in the absence and the presence of humic acids, respectively. The measurements are not significantly different ($\alpha < 0.05$), which was expected if SPME determines the freely dissolved fraction. We can therefore state that humic acids do not influence the SPME measurements and that negligible depletion SPME measures the free concentration in the presence of humic acids. Since PCB 77 is one of the most hydrophobic chemicals under study, effects are not likely for the other compounds. In addition, protection of the fiber recommended by Zhang et al. (*30*) does not seem to be necessary for short sampling times as used in this study.

***K_{DOC}* Determination.** The reduction of the free fraction of the dissolved compound, as measured using nd-SPME, in the presence of HA is shown in Figure 2. Fitting of these experimental data to eq 2 resulted in the log K_{DOC} values listed in Table 2. Values for log K_{DOC} obtained in these experiments are in excellent agreement with the values reported previously in the literature. Looking at the replicates, Figure 2 shows that in general the accuracy of the measurements is high. However, it is also observed that the spread between a few replicates is very large. These measurements are all at low HA concentrations, where the curves show steep slopes (high differences in the free fraction are achieved by a small variation in the amount of dissolved humic acids). The slope in this area is highly determined by the partition coefficients (K_{DOC}). Higher coefficients lead to steeper slopes, because less HA is needed to reduce the free fraction. As a consequence, larger errors are found for more hydrophobic chemicals, and therefore the estimates of K_{DOC} values for these chemicals are less reliable (see Table 2).

These values are based on the manual sampling system. As we have discussed above, the rate-limiting step in this system is assumed to be the diffusion through the stagnant

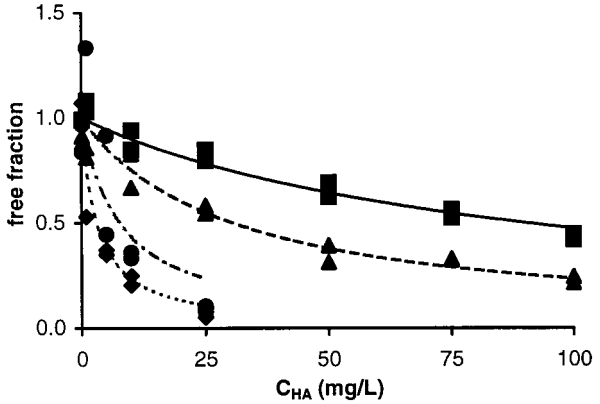

FIGURE 2. Free fraction determined using SPME vs the humic acids concentration in water. The lines represent eq 2 fitted to the experimental values. (■ —, pentachlorobenzene; ▲ — —, hexachlorobenzene; ♦ - - -, PCB 77; ● — - —, DDT).

aqueous layer surrounding the fiber. The presence of this layer may disturb the measurement. The autosampler system seems to achieve 'perfect' stirring (kinetics are determined by diffusion in the polymeric phase). To further validate the determined values, K_{DOC} values determined by both systems were compared for one of the chemicals. DDT was selected for this purpose because this is the most hydrophobic chemical in this study. If effects exist, they should increase with increasing hydrophobicity of the chemicals. Therefore, if no effects are observed with DDT, they are not likely to occur with less hydrophobic compounds. Note that DDT has the slowest absorption kinetics for manual sampling (see Table 1). In this experiment, the same solutions as for manual sampling were used, but the SPME measurements were performed using the autosampler (12 mL of sample, 1 mm long fiber, and 1 min sampling). The obtained value of 5.56 is identical to the one obtained in the manual stirring system (5.57). This suggests that the diffusion from the bulk solution to the stagnant layer is faster than the desorption of the bound fraction already present in the stagnant layer. Therefore, it can be concluded that, even when stagnant layer diffusion is the rate-limiting step, the measurements are not affected by this.

Bioavailability. The bioconcentration experiments with *D. magna* showed that the total concentrations (determined using hexane extraction) were the same in both aquariums (with and without HA): 4.2 and 4.0 μg/L for PeClBz and 1.09 and 1.28 μg/L for PCB 77, respectively. However, as expected, the bioaccumulation in the organisms was lower in the aquarium containing HA. As shown in Figure 3, lower bioconcentration factors were calculated for the HA-containing solution when BCF was based on total aqueous concentration. These results are in agreement with previous studies (*7*−*13*). The differences are not significant for both chemicals, but this can be explained because of the large uncertainty in the BCFs due to the biological variation among individuals. The free concentrations, as measured using nd-SPME, were lower in the aquaria containing HA: 0.69 and 1.20 μg/L for PeClBz and 2.3 and 4.8 μg/L for PCB 77 for samples with and without HA, respectively. By determining the BCFs based on free concentrations, the same values are found for both aquaria, regardless the presence of humic acids (see Figure 3). In addition, these BCFs are also equal to the values obtained based on total concentration in the absence of HA. The results show that the lowering in bioaccumulation can be completely explained and corrected by measuring free concentrations.

General Remarks. We realize that Aldrich humic acids are not comparable with natural humic acids, but this study focuses on the method itself and its applicability for

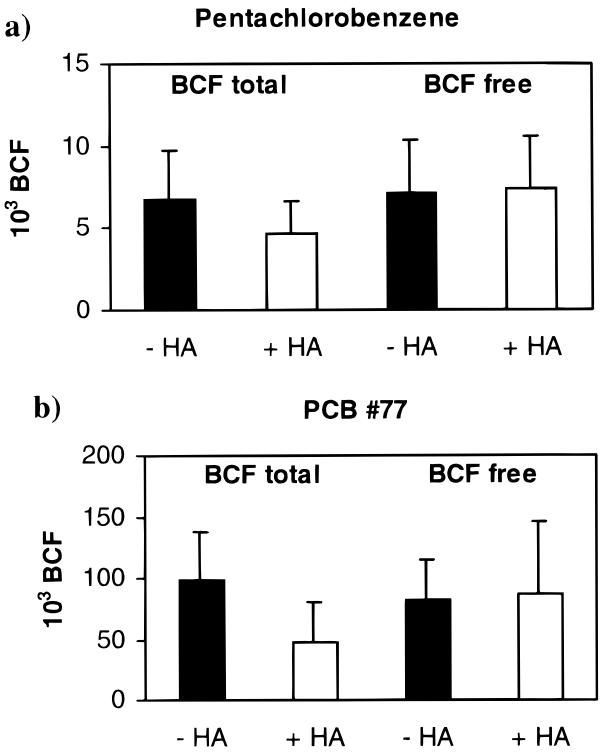

FIGURE 3. Bioconcentration factors of pentachlorobenzene (a) and PCB 77 (b) to *Daphnia magna* based on free and total concentrations in the presence and absence of humic acids.

environmental studies. By minimizing the extraction efficiency, solid-phase microextraction is a useful tool for determining free concentrations in water samples. Although the requirement of the method is that the extraction is negligible, the method is as sensitive as other techniques because the whole extracted amount is used for analysis (injected on the GC). The advantages of nd-SPME over other techniques include being fast, involving minimal manipulation of the sample, and being easily automated. This method can be used to easily determine partition coefficients of chemicals to environmental matrix components (e.g., DOC, sediments), which are necessary to assess the environmental fate of pollutants. Moreover, the method is useful to monitor the bioavailable concentrations in natural waters as well as in toxicological tests by determining the activities at which effects are achieved.

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