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Biomimetic Extraction as a Tool To Identify Chemicals with High Bioconcentration Potential: An Illustration by Two Fragrances in Sewage Treatment Plant Effluents and Surface Waters

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The Empore disk biomimetic extraction procedure is a method to estimate total body residues (TBR_{est}) in biota after exposure to complex mixtures of organic chemicals in water. Except for highly hydrophobic compounds, the extraction procedure is nondepletive by using an excess of water. Therefore, it is a selective extraction process of the bioavailable fraction of compounds. The extent, to which compounds are extracted, depends on their hydrophobicity. Consequently, compounds that only have a minor contribution to the total amount in exhaustive extracts can become very prominent in the biomimetic extracts. Bioconcentration is also a process that depends primarily on hydrophobicity. In this study the method is applied to selectively focus on compounds with a high bioconcentration potential. This feature is illustrated by data on two fragrances (HHCB and AHTN) in effluents of municipal sewage treatment plants and several types of surface water. Although estimated aqueous concentrations of both AHTN and HHCB ranged from about only 1 ng/L in clean surface water to 500 ng/L in the effluents of sewage treatment plants, the contribution of these two compounds together to the total amount of extracted compounds varied from 1 to 23% for surface waters and from 5 to 22% for effluents.

Introduction

Bioaccumulation of organic trace pollutants into organisms leads to certain internal concentrations. The sum of the internal concentrations of all compounds is referred to as the total body residue (*TBR*), which is a measure of total bioaccumulation and predicts baseline toxicity due to narcosis (*1*−*3*). Biomimetic extraction is a technique to estimate *TBR*s in biota after exposure to complex mixtures of organic chemicals in water.

In a standard exhaustive extraction, polar and hydrophobic chemicals will be extracted from the aqueous phase nearly quantitatively, and, therefore, all these compounds will accumulate on the hydrophobic phase to the same extent. In a biomimetic extraction, the more hydrophobic chemicals will be extracted more efficiently than less hydrophobic chemicals, in a similar way as chemicals (if they are not rapidly metabolized in biota) are concentrated from the aqueous phase by bioconcentration (*1*−*3*). Moreover, the biomimetic extraction only takes into account the bioavailable fraction in the aqueous phase of the water samples, while an exhaustive extraction also includes the fraction of the chemical associated with suspended particles. To extract compounds in a hydrophobicity dependent, biomimetic manner, the depletion of the organic compounds in the aqueous phase during extraction should be as low as possible for all compounds. This condition of minimal depletion leads to specific requirements on the volume ratio of both phases (*1*): the volume of the hydrophobic phase has to be very small compared to the volume of the aqueous phase.

The biomimetic extraction procedure selectively recovers compounds that can contribute to the total body residue in biota due to their high $\log K_{ow}$. Such hydrophobic compounds, because of their generally higher bioconcentration factor, can make a small contribution to the total amount of organic compounds in water but a major contribution to the total body burden. Therefore, this extraction method is a suitable tool to screen for compounds that might may give rise to high body residues in biota.

Different hydrophobic phases have been used to simulate uptake from water into biota. Södergren (*4*) and Huckins (*5*) have used semipermeable polymeric membrane devices (SPMD) to measure the freely dissolved concentration of organic micropollutants. In this method a dialysis membrane is filled with an apolar solvent, e.g. hexane or triolein. Recently, Empore disks, containing C_{18} material, have been used for this purpose (*1*−*3*). The possibility to measure only the freely dissolved, bioavailable concentration of organic compounds by means of extraction with Empore disks has been demonstrated by Freidig et al. (*6*). Other methods to measure the freely dissolved concentration of chemicals include solid-phase microextraction (SPME, (*7, 8*)).

There are several advantages of the use of hydrophobic C_{18} material or other hydrophobic materials over an analysis in biota. First, no clean up of the extracts is needed, because there is less interference of impurities in samples, such as lipids and proteins in biotic extracts. Second, the extraction procedure is relatively fast and less expensive without a need for testing animals. A disadvantage of the procedure is that it overestimates bioconcentration of compounds that are biotransformed rapidly.

Another approach to examine organic compounds in industrial effluents with a high contribution to the total body burden in biota was given by Hynning (*9*) and Klamer and Beekman (*10*). This approach includes three steps: first, the water sample is extracted by hexane/*tert*-butyl methyl ether or hexane/acetone; then the mixture is separated on a reversed phase HPLC system; and finally the compounds in the fractions of different hydrophobicity are identified. Although these methods deal with the differences in hydrophobicity in a mixture, they are based on exhaustive extractions.

In this study, the biomimetic extraction approach is illustrated by an examination of the presence of HHCB

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(1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-γ-2-benzopyran) and AHTN (6-acetyl-1,1,2,4,4,7-hexamethyltetraline), both in effluents of sewage treatment plants and in surface waters in The Netherlands. HHCB and AHTN belong to the group of polycyclic musks, which are used as fragrances in cosmetics and detergents for household purposes. Polycyclic musks were already determined in human adipose tissue (*11*−*13*) and human milk (*11, 12*), probably as a consequence of dermal absorption due to the use in cosmetics. In addition, because of the use in detergents these compounds can be expected in municipal wastewater treatment plants. Because of the high hydrophobicity of these compounds, it is expected that they will be magnified in the biomimetic extracts. In exhaustive extractions of surface water samples in domestic regions and effluents of sewage treatment plants, these compounds are only present in low concentrations (0.1−1 μg/L) (*14*). As mentioned by van Loon et al. (*3*), the compounds HHCB and AHTN are major compounds in the biomimetic extracts of the effluents of some sewage treatment plants in The Netherlands.

Experimental Section

Sampling. Effluent samples (2 × 10 L; $n = 10$) were provided in winter 1995/1996 by the Institute for Inland Water Management and Waste Water Treatment (RIZA, The Netherlands). The effluents, which are kept anonymous in this paper, were sampled from chemical industries (n=5), paper industries (n=2), and sewage treatment plants (n=3), respectively. These sampling locations cover many types of effluents encountered in The Netherlands. These samples were provided before and after an additional biodegradation step of 28 days by an inoculum from lake Markermeer (NL) (*15*). Furthermore, a sample from one additional sewage treatment plant was taken.

Surface water samples (2 × 10 L, n=12) were taken in spring 1995 from the river Rhine at Lobith (NL) in duplicate, the river Meuse at Eijsden (NL) and Luik (B), the river Eem (NL), the river Drentsche Aa (NL), the river Scheldt (B), lake IJsselmeer (NL), lake Ketelmeer (NL), lake Markermeer (NL), the North Sea at Scheveningen (NL), the Wadden Sea at Pieterburen (NL), and the Westerscheldt estuary at Kruiningen (NL). The sampling locations cover most surface water types encountered in The Netherlands. To minimize adsorptive losses of HHCB and AHTN onto the glass wall of the sample bottle, the latter was rinsed three times with the water sample prior to filling the bottle in order to saturate adsorption sites. The samples were stored at 4 °C under dark conditions to prevent algal growth.

Materials. Chemicals used are cyclohexane (J. T. Baker, Deventer, The Netherlands, resi analyzed), 2,4,5-trichlorotoluene (Janssen Chimica, Geel, Belgium, 98%), silver nitrate (J. T. Baker, Deventer, Baker analyzed), HHCB (International Flavors and Fragrances-IFF, Hilversum, The Netherlands B. V., CAS no. 1222-05-5), and AHTN (PFW Aroma Chemical B. V., Hercules Incorporated, Barneveld, The Netherlands, >98%, CAS no. 1506-02-1 or 21145-77-7). Empore disks (C_{18} solid phase; ∅47 mm; 90% w/w octadecylsilica of which 17% w/w organic carbon, 10% w/w Teflon fibers, J. T. Baker) were used. Homemade (RITOX, The Netherlands) Empore disk holders were used. A gas chromatography−mass spectrometry (GC-MS) combination from Carlo Erba Instrument (Milan, Italy), consisting of a Model OC516 on-column control unit, a Model MFC500 gas chromatograph, and a Model QMD-1000 quadrupole mass spectrometer, was used for the identification and quantification of major bioaccumulatable compounds.

Methods. Biomimetic extractions were carried out for all samples. In addition, for most effluents exhaustive extractions were performed. The biomimetic extraction and total molar determination procedures are described in detail elsewhere (*1, 2*). A short description will be given here.

The Empore disks were cleaned thoroughly (*2*) prior to use. A biomimetic extraction was performed in a 10 L glass bottle by stirring (300 rpm) a water sample (10 L), in which a ∅ 13 mm (∼ 48 mg ≅ 11 μL C_{18}) Empore disk was placed in a disk holder at 25 °C under dark conditions to prevent algal growth. The corresponding phase ratio C_{18}:water was about 1:10^6. To the water sample, 5 mL of a silver nitrate solution (1 mg/mL) was added to prevent bacterial growth. After 7 days, the water sample was refreshed and a total partitioning time of 14 days was allowed. Then, the disk was removed from the water sample, suspended matter was wiped off by a tissue, and the disk was inserted immediately into 2 mL of cyclohexane. Aquatic humic and fulvic acids do not bind to the Empore disk significantly, and therefore do not disturb the procedure (*2, 6*). After an extraction time of 1 day, the Empore disk was removed from cyclohexane. As shown in previous work, recoveries of extraction of hydrophobic chemicals from the Empore disk are higher than 95% (*2*).

The exhaustive extractions were performed using entire Empore disks (∅47 mm; ∼ 0.6 g ≅ 145 μL C_{18}). In this case, the phase ratio C_{18}:water was about 1:70. The disks were placed in a Petri dish and were kept to the bottom of the dish by plastic pipet tips. To these Empore disks 10 mL of a sample and 10 μL of the silver nitrate solution were added. Every 2 days, the water sample was refreshed to a total of four times. After 8 days, the Empore disk was removed from the water sample, suspended matter was wiped off by a tissue, and the disk was inserted immediately into 25 mL of cyclohexane. After the extraction time of 1 day, the Empore disk was removed from cyclohexane.

Total molar determinations using GC-MS were performed as follows. First, a GC-MS total molar determination was performed on the 2 mL cyclohexane extract to determine if concentration of the extract by evaporation was necessary and, if so, the extract was concentrated 10 times by evaporation under nitrogen. The volume of the concentrated extracts was determined gravimetrically. It was reported for the present evaporation procedure that the evaporation recovery of 1,2,3,4-tetrachlorobenzene is quantitative (99%). Even for the relatively evaporable compound 1,4-dichlorobenzene, the evaporation recovery was 87% (*2*). The vapor pressures of these compounds are 5 and 90 Pa at 25 °C, respectively (*16*). In view of the vapor pressures at 25 °C of HHCB (0.0727 Pa) and AHTN (0.0608 Pa) (*14*), the evaporation recoveries of these two fragrances in the present procedure will probably be quantitative. Therefore, the evaporation recoveries of HHCB and AHTN were not determined experimentally anymore.

Then, the internal standard (2,4,5-trichlorotoluene) was added to the extract, either by injection of 2 or 20 nmol in 5 μL to the evaporated sample or by adding 100 μL of the internal standard solution (containing 4 nmol) gravimetrically to 500 μL of the sample.

The samples were injected on-column. The injection volume was 5 μL. A secondary cooling time of 10 s was used. The analysis of the samples was carried out using a deactivated fused silica retention gap (J&W, Folsom, CA, U.S.A., length 5 m, ID 0.32 mm) connected with a press fit to a short column (J&W DB-1, length 5 m, ID 0.32 mm, film thickness 0.1 μm). A rapid temperature program starting at 40 °C for 2 min to 290 °C at a speed of 30 °C/min was used. The temperature of 290 °C was achieved at 13 min (the end of the total run time).

The used MS-mode was full scan with a scan range of 34−434 or 34−500 m/z. The cycle time was 0.5 s with 0.05 s as interscan delay. Helium was used as carrier gas with a flow rate of ca. 1 m/s (determined by injection of 5 μL of an

argon/methane mixture at 175 °C or 0.1 μL cyclohexane at 100 °C). Duplicate determinations were carried out. The relative molar GC-MS responses of HHCB and AHTN were determined by injection of a mixture of HHCB, AHTN, and 2,4,5-trichlorotoluene in cyclohexane.

Calculations. Total molar concentrations in cyclohexane, on the Empore disk and the estimated *TBR*, respectively, were calculated using equations derived and reported elsewhere (*1*, *2*). The molar responses of the two compounds relative to the internal standard 2,4,5-trichlorotoluene were used for the determination of the concentrations in the extracts. For HHCB and AHTN, the concentrations in the effluents of three sewage treatment plants and surface waters were calculated from biomimetic extractions considering the following aspects.

If HHCB and AHTN were not completely separated on the GC, the concentrations of the two compounds were calculated from the ratios of the following selective ions of HHCB and AHTN: m/z 43, 143, 159, 171, 187, 213, 243, 244, and 258.

Values for log K_{ow} were used to estimate the ratio between the concentrations of compounds on the disk and in the aqueous phase. The used values for log K_{ow} of HHCB and AHTN were 5.9 and 5.7, respectively (*14*). Equilibrium partition coefficients between Empore disk and water (log K_d) were estimated by the equation given by Verhaar et al. (*1*):

$$\log K_d = 0.995 \cdot \log K_{ow} + 0.70 \quad (1)$$

This equation was derived for a set of chemicals with diverse structures (*1*). However, for hydrophobic compounds, the fraction of equilibrium reached after two weeks is substantially less than unity (*1*). Partitioning to the Empore disk follows first-order kinetics. The first-order rate constants for uptake and elimination by the Empore disk (k_1 and k_2) were estimated from the uptake rate constants for five other compounds of both lower and higher log K_{ow} (*6*) and the equilibrium disk partition coefficients, assuming that the partition coefficient K_d is equal to k_1/k_2. The uptake rate constants obtained in this way were 4930 and 4990 $L_a/(L_d \cdot h)$, and the elimination rate constants were 0.0013 and 0.0021 h^{-1} for HHCB and AHTN, respectively. If no significant depletion of the compounds in the aqueous phase occurs, it is allowed to use the following equation to calculate to what extent equilibrium is achieved:

$$C_{d,t} = C_a \cdot \frac{k_1}{k_2} \cdot (1 - e^{-k_2 \cdot t}) \quad (2)$$

In this equation, t is the time of partitioning process (336 h), $C_{d,t}$ is the concentration on the disk at time t [ng/L_d], and C_a is the concentration in the aqueous phase [ng/L_a].

After two weeks of exposure 36% of equilibrium is achieved for HHCB and 51% for AHTN, assuming that no depletion occurs.

Although more than half of these compounds may be extracted at equilibrium, still a considerable amount of the compounds will be present in the aqueous phase after two weeks. Further, the suspended particles in the effluent samples will likely release the compounds partly if the concentration in the aqueous phase decreases. However, data on kinetics of these processes are not available, and therefore this process could not be modeled. Finally, after 1 week the effluent sample is refreshed to minimize depletion. Considering these facts, depletion will probably be low, and eq 2 is used to calculate the concentrations of HHCB and AHTN in the aqueous phase of the water samples.

However, if depletion is fully taken into account, the concentrations on the disk can be calculated using the following equation:

$$C_{d,t} = C_{a,0} \cdot \frac{k_1}{m} \cdot (1 - e^{-m \cdot t}) \quad (3)$$

In this equation, $C_{a,0}$ is the initial concentration in the aqueous phase and m is a constant defined as

$$m = k_2 + k_1 \cdot \frac{V_d}{V_a} \quad (4)$$

Here, V_d and V_a are the volumes of the hydrophobic C_{18} phase and the aqueous phase, respectively. The concentration on the disk after 1 week can be calculated by eq 3. It should be considered that the water sample is renewed after 1 week. So, after 1 week the initial concentration in the aqueous phase is restored, and the concentration on the disk is equal to the concentration on the disk at the end of the first week. In this case, the following equation can be used to calculate the amount on the disk after the second week:

$$C_{d,t} = \frac{n \cdot (1 - e^{-(m \cdot t)}) + m \cdot C_{d,0} \cdot e^{-(m \cdot t)}}{m} \quad (5)$$

In this equation, $C_{d,0}$ is the initial concentration in the hydrophobic C_{18} phase (the concentration on the disk after 1 week) and n is a constant defined as

$$n = \left(C_{a,0} + C_{d,0} \cdot \frac{V_d}{V_a} \right) \cdot k_1 \quad (6)$$

In this case, the concentrations on the disk after 2 weeks are equal to 25% and 36% for HHCB and AHTN, respectively, of the concentrations calculated from the partition coefficient, in which both nonequilibrium and depletion are neglected. This means that the concentrations of both compounds in the aqueous phase, estimated from eq 2, are underestimated by a factor 1.4, if the release of the chemicals from the suspended particles during extraction is completely negligible.

Results and Discussion

Biomimetic vs Exhaustive Extractions. The biomimetic extraction procedure selectively extracts compounds from the aqueous phase: compounds with high hydrophobicity are extracted more effectively in a similar way as in the bioconcentration process. This implies that the compounds in exhaustive extracts with the highest concentrations are different from those in the corresponding biomimetic extracts. This phenomenon is indeed observed in Figure 1, in which the composition of the biomimetic extracts of the same water samples is compared to that of the exhaustive extracts. Completely different chromatographic profiles are observed, showing the different selectivity of the biomimetic versus the exhaustive extraction procedure. The biomimetic extraction can be used to identify the compounds that have a high potential for bioaccumulation, and examples are the two identified compounds, AHTN and HHCB.

Besides the presence of the compounds HHCB and AHTN in the biomimetic extracts of effluents of sewage treatment plants (*3*), these compounds were also observed in the biomimetic extracts of all analyzed surface waters at relatively high concentrations (see for example Figure 2). On the contrary, in the exhaustive extracts of the same samples these compounds were not detected. As expected, these compounds were not present in the extracts of industrial effluents nor in blank disks.

Quantitative Determination of AHTN and HHCB. The detection limits (S/N-ratio = 10) for HHCB and AHTN in

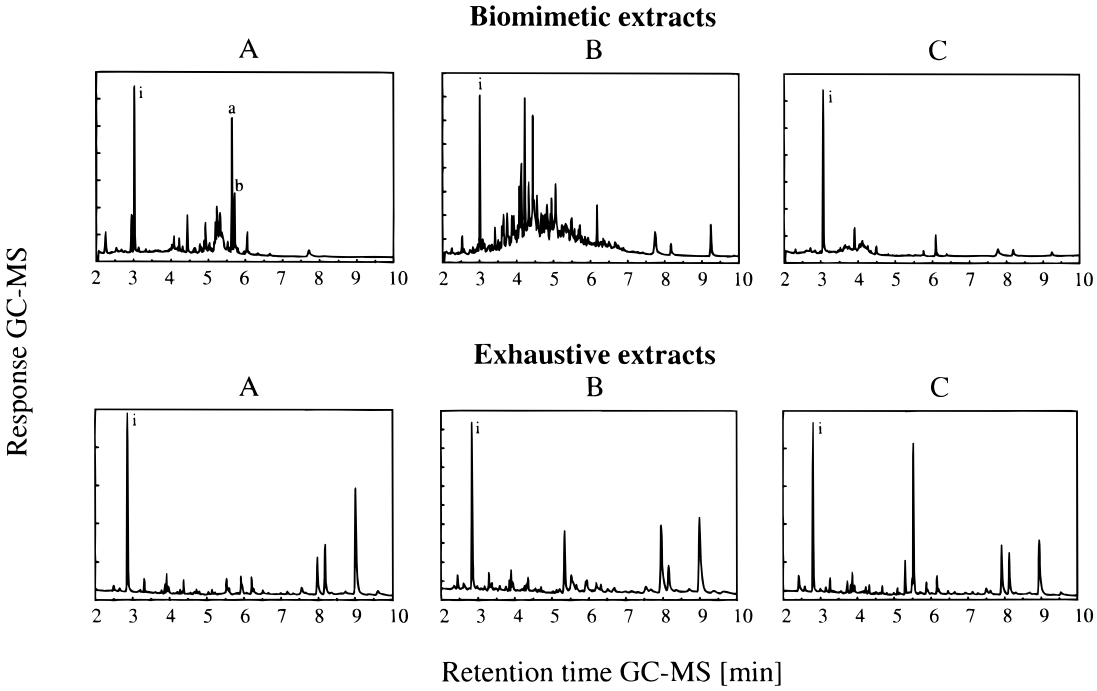

FIGURE 1. Biomimetic extracts vs exhaustive extracts: (A) effluent of sewage treatment plant 3 and (B and C) industrial effluents, (i) internal standard (2,4,5-trichlorotoluene), (a) HHCB and (b) AHTN. Note the absence of the two peaks of HHCB and AHTN from the exhaustive extract of the sewage sample. The three peaks between 7.5 and 9.5 min are present in all exhaustive extracts and are probably contaminations.

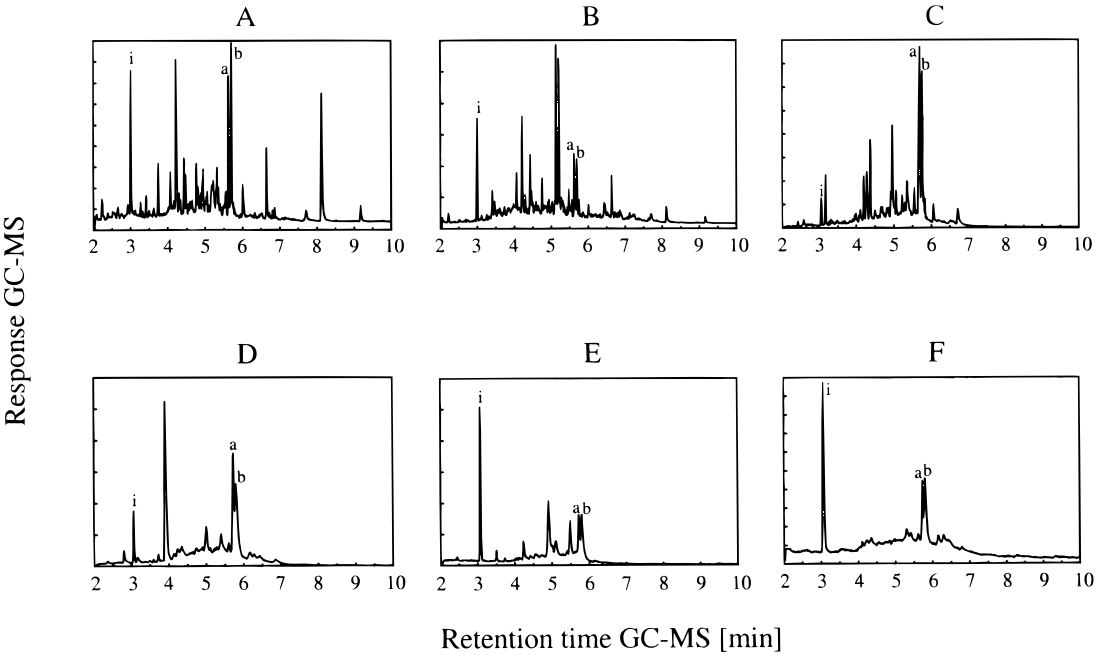

FIGURE 2. Biomimetic extracts of effluents of sewage treatment plants and of surface waters, showing the ubiqitous presence of HHCB and AHTN in sewage, surface, and seawater: (A) effluent of sewage treatment plant 1, (B) effluent of sewage treatment plant 2, (C) effluent of sewage treatment plant 4, (D) river Eem, (E) lake Ketelmeer, and (F) North Sea at Scheveningen.

water were calculated to be 0.1 ng/L, based on the ion mass of m/z 243. These very low detection limits illustrate the potential of the biomimetic extraction procedure to detect hydrophobic compounds. The detection limit of the biomimetic extraction/GC-MS procedure is a function of the hydrophobicity of a compound. The linear dynamic range of the GC-MS determination was reported to be 1−500 ng (*2*). The repeatability of the GC-MS determination is illustrated by the duplicate Rhine water sample. The relative molar response factors of HHCB and AHTN were 1.26 and 0.94, respectively. Using these molar response factors the free concentrations of these compounds (concentration in the aqueous phase) were estimated by eqs 1 and 2. These estimated concentrations are dependent on log K_{ow} of the compounds. Uncertainty in the experimental values of log K_{ow} of HHCB and AHTN will lead to an error in the estimated aqueous concentration of both compounds. However, from eqs 1 and 2 it can be derived that the relative amount accumulated on the disk after 2 weeks, increases slightly for log K_{ow} values between 5 and 7. The corresponding calculated aqueous concentration is therefore subject to only small errors: the calculated aqueous concentration increases less

TABLE 1. Estimated Free Concentrations of HHCB and AHTN in the Sewage Treatment Plant Effluents and Surface Waters, the Estimated Total Body Residues (TBR_{est}), and the Percentages Contribution of HHCB and AHTN to These Total Body Residues

sampling location	C_a[d] [ng/L] HHCB	C_a [ng/l] AHTN	TBR_{est} [mmol/kg]	% of TBR_{est} HHCB	% of TBR_{est} AHTN
STP[a] 1	279	417	5.07	5.6	7.5
STP 2	162	156	6.34	2.6	2.3
STP 3	219	109	1.48	15.2	6.8
STP 4	629	424	6.42	10.0	6.1
STP 1 biodegraded[b]	5.7	79	0.84	0.7	8.6
STP 2 biodegraded	2.1	11	0.64	0.3	1.6
STP 3 biodegraded	1.8	4.6	0.20	0.9	2.1
Eem, Baarn	182	175	1.51	12.3	10.7
Drentsche Aa	0.5	2.7	0.24	0.2	1.0
Ketelmeer	27	34	0.34	8.2	9.4
Markermeer	0.6	2.8	0.17	0.4	1.5
North Sea, Scheveningen	20	29	0.26	7.8	10.3
Rhine, Lobith[c]	26	55	0.35	7.8	14.5
Rhine, Lobith[c]	30	51	0.33	9.2	14.1
Meuse, Eijsden	84	145	1.03	8.4	13.0
Meuse, Luik	36	82	1.36	2.7	5.5
Scheldt, Antwerp	149	124	2.65	5.8	4.3
Meuse, Eijsden	35	87	0.96	3.8	8.4
Westerscheldt, Kruiningen	12	19	0.07	2.6	3.7
Wadden Sea, Pieterburen	0.6	1.2	0.47	0.8	1.6
IJsselmeer	0.6	1.6	0.09	0.7	1.7

[a] STP: Sewage treatment plant. [b] Effluents are exposed for 28 days to an inoculum from lake Markermeer (*15*). The sample is diluted twice by the addition of the inoculum, and hence, concentrations are lowered twice. [c] Duplicate sample. [d] C_a: concentration in aqueous phase estimated by eq 2.

than 50% for a log K_{ow} value, which is 0.5 unit lower, and decreases less than 25% for a log K_{ow} value, which is 0.5 unit higher, for both AHTN and HHCB. The uncertainty in the estimated aqueous concentrations from biomimetic extracts is therefore probably less than a factor 2 for these compounds.

The concentrations of HHCB and AHTN in the sewage treatment plant (STP) effluents and surface waters are listed in Table 1. As can also be seen from this table, the compounds HHCB and AHTN form an important part of the estimated *TBR*. Only in the less polluted surface waters (river Drentsche Aa, Wadden Sea at Pieterburen, lake IJsselmeer and lake Markermeer) the total contribution of the two compounds to the estimated *TBR* is less than 2.5%. Probably, there are no direct or nearby emissions of municipal wastewater treatment plants into these surface waters. However, even in the North Sea at Scheveningen, AHTN and HHCB are present in considerable amounts, probably due to the discharge of the large rivers, Rhine, Meuse, and Scheldt. Because the samples cover most surface water types encountered in The Netherlands, the data from Table 1 indicate that HHCB and AHTN are widely distributed in the Dutch aquatic environment.

Total vs Freely Dissolved Concentrations of HHCB and AHTN. If no depletion occurs, the biomimetic extraction measures only the freely dissolved, bioavailable concentration in the aqueous phase. The aqueous concentrations of HHCB and AHTN in the most polluted rivers approach the concentrations of the effluents of municipal sewage treatment plants (Table 1), although the dilution of these effluents is large after emission into surface waters. These high freely dissolved concentrations in rivers can be explained by the desorption of the compounds from suspended particles present in the effluents. Effluents from a sewage treatment plant have a concentration of suspended particles in the order of 40 mg/L (*17*), which is only slightly higher (about a factor 2) than the density of suspended particles in Dutch rivers. However, the suspended particles in effluents of sewage treatment plants have an organic carbon content that is at least 10 times higher than that of suspended particles in natural aquatic systems (*17*). Therefore, the fraction of the chemicals associated to the suspended particles will be much higher in the effluents of a sewage treatment plant than in river water. For the logarithm of the sorption coefficient normalized to the fraction organic carbon of the sorbent (K_{oc}) values ranging from 4.85 to 4.86 for HHCB and from 4.74 to 4.80 for AHTN were reported (*14*). Assuming that the fraction organic carbon (f_{oc}) of these particles is 50% and using these values for log K_{oc}, it can be calculated that more than half of these compounds is associated with suspended particles. If the effluents are mixed with water containing much less organic carbon in suspended matter, the adsorbed chemical will be partly released. In view of these considerations, the total concentration in the effluents of the sewage treatment plants is expected to be higher than the concentration in the aqueous phase only. This is in agreement with data for concentrations of HHCB and AHTN in effluents of sewage treatment plants. By exhaustive extraction, concentrations ranging from 600 to 2500 ng/L for HHCB (median 1200 ng/L) and 800 to 3100 ng/L for AHTN (median 1600) were found in effluents of sewage treatment plants in Germany (*18, 19*). Polycyclic musks were also identified in effluents of some large wastewater treatment plants in Sweden (*20*), containing total amounts of HHCB between 1 and 6 μg/L. These concentrations determined by exhaustive extraction are approximately five times higher than the freely dissolved concentrations in effluents of sewage treatment plants from Table 1.

In the river Ruhr in Germany, concentrations ranging from 100 to 500 ng/L (median 500 ng/L) and from 100 to 300 ng/L (median 200 ng/L) were found, for HHCB and AHTN, respectively (*14, 18, 19*). In Switzerland, concentrations of 136 ng/L for HHCB and 75 ng/L for AHTN were reported in the river Glatt nearby a sewage treatment plant (*13*). These concentrations in river water are higher than the aqueous concentrations in most of the polluted rivers in this study (Table 1). However, these concentrations from literature are concentrations in exhaustive extracts, including suspended matter, while the concentrations from this study reflect the freely dissolved, bioavailable concentrations. Water samples from the rivers Meuse and Rhine in The Netherlands, which were filtered before extraction, showed lower concentrations of both AHTN and HHCB and they agree with the data

presented in this study. The median concentrations were 60 and 80 ng/L for HHCB and 50 and 70 ng/L for AHTN, in the river Rhine and Meuse, respectively (*14*). This shows the ability of the biomimetic extraction procedure to determine the bioavailable, freely dissolved concentration. The fraction of the compound associated with particulate matter is essentially not extracted.

Aquatic Toxicity, Biotransformation, and Bioconcentration of HHCB and AHTN. A potential risk of the chemicals HHCB and AHTN strongly depends on toxic effect concentrations. Effect concentrations for aquatic organisms (Algae (*Pseudokirchneriella subcapitata*) 72 h; *Daphnia magna* 21 days; Bluegill sunfish (*Lepomis macrochirus*) 21 days; and Fathead minnow (*Pimephales promelas*) 36 days, early life stage) appear to be between 0.1 and 1 mg/L for both HHCB and AHTN (*14*). Effect concentrations, estimated by QSARs for narcosis based on hydrophobicity, are in the order of 0.01 to 1 mg/L (*21*−*23*). Experimental effect concentrations for similar effects are generally slightly higher than these estimates.

Biotransformation of these compounds may reduce the bioconcentration factors, and this may result in effect concentrations that are higher than expected from baseline toxicity (*14*). The concentrations in fish would also be lower than concentrations in fish estimated by the biomimetic extraction procedure. Reported BCF values of HHCB and AHTN are lower than is expected on the basis of their log K_{ow} values: in Bluegill sunfish (*Lepomis macrochirus*) $BCF_{wet\ weight}$ = 1584 L/kg for HHCB and 597 L/kg for AHTN, and it was shown that polar metabolites were formed (*14*). It is apparent from Table 1 that in the samples close to the source, concentrations of HHCB are mostly higher than concentrations of AHTN, while in remote areas concentrations of AHTN are higher. This effect cannot be explained by the higher hydrophobicity of HHCB, because this would lead to a higher value of the ratio HHCB/AHTN after dilution with water containing less organic carbon in suspended particles. However, from the biodegradability studies of the effluents (Table 1), it can be concluded that the decrease of the ratio of HHCB/AHTN might be assigned to the higher biodegradability of HHCB. In the biodegradability test (*15*), the effluents are diluted twice by the addition of the inoculum. The decrease of the concentrations of AHTN but particularly of HHCB is much more than 2-fold (see Table 1). Although the concentrations of both compounds obviously decreased after the biodegradation step, mineralization of HHCB and AHTN in water appeared to be insignificant in several other biodegradability tests (*14*). Also the occurrence of these compounds in all types of surface water suggests that these compounds are not very rapidly degraded in the aquatic environment (Table 1).

Experimental body residues of AHTN and HHCB in pelagic fish from the river Ruhr in Germany (1−7 mg/kg_{lipid}) (*19*) are within the range of the estimated body residues from the Empore disk extracts (0.1−50 mg/kg_{lipid}) of Dutch and Belgium rivers. Experimental body residues in pelagic fish from an effluent pond (1−37 mg/kg_{lipid}) (*19*) are in between the experimental body residues form the river Ruhr and the estimated body residues from the direct effluent of municipal sewage treatment plants in The Netherlands (25−165 mg/kg_{lipid}). It seems that for these compounds, the biomimetic extraction procedure gives good estimates of the accumulation of compounds from environmental water samples into aquatic organisms. However, fish are assumed to be in equilibrium with the water, in which they were caught. Estimated total body residues are directly derived from concentrations on the disk, and after 2 weeks of extraction the concentration on the disk has only reached 25−36% of equilibrium for HHCB and 36−51% for AHTN. Obviously, this leads to an underestimation of the body residue by a factor 2−4.

The uptake to the disk is only physical partitioning, and therefore body residues are overestimated for species in which the compounds are metabolized. Both compounds are subject to biotransformation in bluegill sunfish (*Lepomis macrochirus*), and the bioconcentration factor is 1 order of magnitude lower than log K_{ow} based estimates (*14*). The ability for biotransformation may differ between different species, and consequently the extent to which the bioconcentration factor is overestimated is not known. Nonetheless, it should be noted that the biomimetic extraction procedure represents a worst case for species that are not able to metabolize these compounds. The combined effects of nonequilibrium and metabolism probably partly cancel each other. Therefore, the estimated body burdens from biometic extracts are still comparable to experimental body burdens of HHCB and AHTN for similar water samples.

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