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MEASURING BIOCONCENTRATION FACTORS IN FISH USING EXPOSURE TO MULTIPLE CHEMICALS AND INTERNAL BENCHMARKING TO CORRECT FOR GROWTH DILUTION

MARGARETHA ADOLFSSON-ERICI, GUN ÅKERMAN, and MICHAEL S. MCLACHLAN*
Department of Applied Environmental Science, Stockholm University, Stockholm, Sweden

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Abstract—Modern chemical legislation requires measuring the bioconcentration factor (BCF) of large numbers of chemicals in fish. The BCF must be corrected for growth dilution, because fish growth rates vary between laboratories. Two hypotheses were tested: (1) that BCFs of multiple chemicals can be measured simultaneously in one experiment, and (2) that internal benchmarking using a conservative test substance in the chemical mixture can be used to correct for growth dilution. Bioconcentration experiments were conducted following major elements of the OECD 305 guideline. Fish were simultaneously exposed to 11 chemicals selected to cover a range of BCFs and susceptibility to biotransformation. A method was developed to calculate the growth-corrected elimination rate constant from the concentration ratio of the analyte and a benchmarking chemical for which growth dilution dominated other elimination mechanisms. This method was applied to the experimental data using hexachlorobenzene as the benchmarking chemical. The growth dilution correction lowered the apparent elimination rate constants by between 5% and a factor of four for eight chemicals, while for two chemicals the growth-corrected elimination rate constant was not significantly different from zero. The benchmarking method reduced the uncertainty in the elimination rate constant compared to the existing method for growth dilution correction. The BCFs from exposing fish to 10 chemicals at once were consistent with BCF values from single-chemical exposures from the literature, supporting hypothesis 1. Environ. Toxicol. Chem. 2012;31:1853–1860. © 2012 SETAC

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INTRODUCTION

The fish bioconcentration factor (BCF) plays an important role in the regulatory assessment of chemicals. It is measured in laboratory experiments in which the uptake and elimination kinetics of the chemical are determined by exposing a group of fish to water containing the chemical for several weeks and then transferring them to chemical-free water for an additional several-week depuration period. Fish are killed at different time points throughout the experiment and analyzed for chemical residues. These experiments are costly and require large numbers of fish. OECD 305 guideline for bioconcentration in a flow-through fish test, a recognized standard method for determining the BCF, requires on the order of 100 fish and an experiment that can last several months, whereby only one chemical is studied per experiment [1]. Recently enacted chemical legislation has increased the requirements for BCF determination. For example, the implementation phase of the European chemicals management program REACH is anticipated to require approximately 65,000 fish for bioconcentration experiments [2]. Hence, there is currently a particularly strong need for more economical means (in terms of both cost and test animals) to determine fish BCFs.

The regulatory use of BCFs requires that the determination be of a high quality. Fish growth has been identified as an important factor that can impact the value of the BCF [3]. The growth of fish leads to a decrease in concentration over time, a phenomenon termed "growth dilution."

All Supplemental Data may be found in the online version of this article.

* To whom correspondence may be addressed (michael.mclachlan@itm.su.se).

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Growth dilution can be viewed as a pseudoelimination process, and when it is large compared to other elimination processes, it results in a lower measured BCF. Fish growth rates can vary widely depending on species and experimental conditions, and they are frequently higher in the laboratory than in the field. Thus, measured BCF values must be corrected for the growth dilution if they are to be comparable between studies. Furthermore, growth-corrected BCF values are required if the experimentally determined values are to be extrapolated to the field. In the research literature, growth dilution has often been estimated using the average change in body mass of the fish cohort over time [4]. However, this method does not account for the individual variability in fish growth rates. The OECD 305 guideline does not foresee a correction for growth dilution.

The present study aimed to address both the need for a more economical determination of the BCF as well as the need for a better method to correct for growth dilution. To reduce costs and animal requirements, a BCF experiment following major elements of the OECD guideline was conducted with simultaneous exposure of the fish to 11 chemicals. Fish BCF experiments using exposure to multiple chemicals have been conducted previously [5,6]. In the present study the BCF values from multiple chemical exposures were compared to BCF values from single-chemical exposures from the literature. To improve the treatment of growth dilution, a benchmarking method was developed that used one of the 11 chemicals as an internal standard.

THEORY

Bioconcentration of organic contaminants in fish is frequently described using a one-compartment model of the

fish and first-order kinetics for chemical exchange between the fish and water. This model is represented by the following differential equation

$$\frac{dC_F}{dt} = k_1 C_W - k_2 C_F \quad (1)$$

where C_F is the chemical's concentration in fish (mol kg^{-1}), C_W is its concentration in water (mol L^{-1}), k_1 is the uptake rate constant (L kg^{-1} d^{-1}), k_2 is the elimination rate constant (d^{-1}), and t is time (d). At steady state this equation reduces to

$$\mathrm{BCF} = \frac{C_F}{C_W} = \frac{k_1}{k_2} \quad (2)$$

This equation is one of the options in the OECD 305 guideline for calculating the BCF; the other being the ratio of the concentration in fish and the concentration in water at steady state [1].

The OECD guideline foresees the use of the total concentration of chemical in water. For hydrophobic chemicals, a considerable fraction of the chemical in the aquarium water can be sorbed to organic material. In such cases the concentration in the water, and hence the BCF, becomes dependent on the amount of organic material in the water and the organic material to water partition coefficient. Because both of these parameters are seldom measured, comparison of measured BCFs between experiments or extrapolation from the laboratory to the field becomes uncertain. From this perspective, it is preferable to use the freely dissolved concentration of chemical in water for BCF determination.

The BCF of neutral organic contaminants is also generally affected by the lipid content of the fish. This is the case when the chemicals are primarily sequestered into the lipid tissue in the fish. A higher lipid content confers a higher capacity to store the chemical, a higher BCF, and a lower elimination rate constant (k_2). To facilitate the comparison of BCF values between studies, the BCF is frequently normalized to a lipid content of 5%.

Another factor that affects the BCF is growth. When the fish are growing, the concentration in the fish can decrease even if there is no elimination of chemical (i.e., k_2C_F in Eqn. 1 is 0). The model including growth is given by

$$\frac{dC_F}{dt} = k_1 C_W - k_2 C_F - k_G C_F \quad (3)$$

where k_G is the rate constant for growth (d^{-1}), that is, for the change of the fish's mass. The equation for the apparent BCF ($\mathrm{BCF_A}$) becomes

$$\mathrm{BCF_A} = \frac{k_1}{k_2 + k_G} = \frac{k_1}{k_T} \quad (4)$$

where k_T is the rate constant for overall elimination including growth. From Equation 4, it is apparent that when k_G is comparable to or greater than k_2, the BCF becomes dependent on the growth rate. This presents a problem analogous to the use of concentrations in bulk water; in order for $\mathrm{BCF_A}$ to be compared with measurements from other experiments, k_G must be known. Alternatively, the results can be presented as growth-corrected BCFs ($\mathrm{BCF_{No\text{-}G}}$, equivalent to BCF in Eqn. 2), which can be directly compared between laboratory experiments and extrapolated to the field using

$$\mathrm{BCF_F} = \mathrm{BCF_{No-G}} \times \frac{k_2}{(k_2 + k_{G,field})} \quad (5)$$

where $\mathrm{BCF_F}$ is the BCF in the field and $k_{G,field}$ is the rate constant for growth in the field.

From Equations 2 and 4 it is apparent that the influence of growth on $\mathrm{BCF_A}$ depends on both the growth rate of the fish and the magnitude of $\mathrm{BCF_{No\text{-}G}}$. Equations 2 and 4 were used to calculate the deviation of $\mathrm{BCF_A}$ from $\mathrm{BCF_{No\text{-}G}}$, whereby a k_1 value of 400 L kg wet weight^{-1} d^{-1} was assumed (Fig. 1). The deviation increased with increasing $\mathrm{BCF_{No\text{-}G}}$. For a fish growth rate of 0.01 d^{-1}, $\mathrm{BCF_A}$ underestimated $\mathrm{BCF_{No\text{-}G}}$ by 11% for a chemical with $\mathrm{BCF_{No\text{-}G}}$ of 5,000, but this increased to 56% (> factor 2) for a chemical with a $\mathrm{BCF_{No\text{-}G}}$ of 50,000.

METHODS

Chemicals

Ethyl acetate (Pestiscan) was purchased from Labscan. Methanol (LiChrosolv), acetone (SupraSolv), and *n*-hexane (LiChrosolv) were from Merck. Sodium chloride, phosphoric acid, and sulfuric acid were from BDH Prolabo. Silicone sheets (0.01-inch thickness) were from Specialty Silicone Products. The SPE cartridges (1 ml 50 mg ISOLUTE ENV +) were from Biotage AB. The water was of Milli-Q grade from a Milli-Q ultrapure water system, MilliQ PLUS 185 (Millipore). The chemicals to which the fish were exposed, the internal standards used for the analysis of these chemicals, and the sources of these chemicals are listed in the Supplemental Data, Table S1.

Experimental procedure

The experiment was based on the OECD 305 protocol for assessing bioconcentration using a flow-through fish test [1]. Fish were placed in an aquarium that was being continuously supplied with water containing the test chemicals. At intervals during this exposure phase, several fish were killed and analyzed for the test chemicals. After 24 d, the remaining fish were transferred to an aquarium that was being continuously supplied with water that was free of the test chemicals. Fish were also killed at intervals during this depuration phase, which was terminated after 56 d. The BCFs were calculated using the kinetic model described above (see *Theory*).

Eleven chemicals were tested simultaneously (see Table 1). They were selected to cover a broad range of BCFs. Both stable substances and substances that are metabolized in fish were included. A further selection criterion was the existence of high-quality data from OECD 305 experiments conducted with single-chemical exposures so that these could be compared

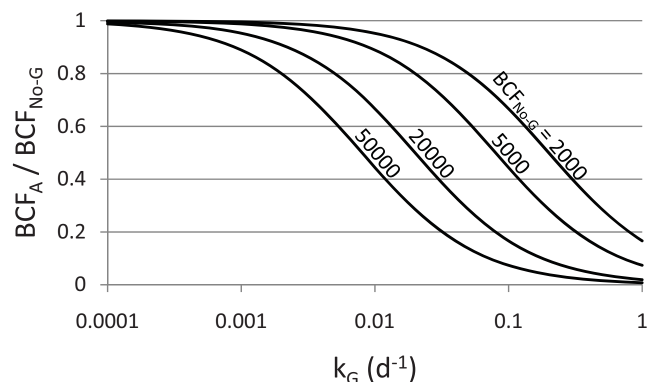

Fig. 1. Model calculations of the deviation of the apparent bioconcentration factor ($\mathrm{BCF_A}$) from the BCF with no growth ($\mathrm{BCF_{No\text{-}G}}$) as a function of the rate constant for growth, shown for chemicals with different values of $\mathrm{BCF_{No\text{-}G}}$.

Table 1. Overall elimination rate constant (k_T), growth-corrected elimination rate constant (k_2), uptake rate constant (k_1), apparent bioconcentration factor (BCF_A), growth-corrected bioconcentration factor ($BCF_{No\text{-}G}$), and bioconcentration factor from the literature (BCF_{LT}) for the test chemicals

				k_T			k_2			k_1	BCF_A	$BCF_{No\text{-}G}$	BCF_{LT}
	CAS no.	Abbrev.	log K_{OW}	Value (d^{-1})	r^2	RSE (%)	Value (d^{-1})	r^2	RSE (%)	(L kg lipid^{-1} d^{-1})	(L kg wet wt^{-1}, 5% lipid)[e]		
2,6-diisopropyl-naphthalene[a]	24157-81-1	DN	6.17[b]	0.090	0.91	6	0.073	0.91	6	8,100	4,500	5,550	4,650[f,g]
2,3,4-trichloroanisole	54135-80-7	ANI	4.1[b]	0.135	0.70	13	0.121	0.67	14	5,500	2,036	2,269	470[h]
p-diisopropylbenzene	100-18-5	ISBE	5.25[b]	0.300	0.87	8	0.285	0.87	8	4,800	801	843	2,566[f]
Musk xylene	81-15-2	MUSK	4.4[b]	0.0328	0.88	6	0.0154	0.91	6	8,900	13,578	28,861	10,015[f]
Chlorpyrifos	2921-88-2	CHPY	5.27[c]	0.109	0.88	7	0.0921	0.90	6	6,600	3,019	3,583	1,687[f,g]
4-*n*-nonylphenol	104-40-5	NP	6.1[b]	0.548	0.66	19	0.461	0.66	19	4,700	431	509	896[h]
2,4,6-tri-*tert*-butylphenol	2051-60-7	BUFE	5.3[b]	0.0189	0.64	14	0.0017	0.01	164	11,500	30,501	340,185	21,778[f]
Pentachlorobenzene	608-93-5	PENT	5.18[c]	0.0233	0.85	7	0.0060	0.73	11	10,700	22,943	89,693	7,600[f]
2,5-dichlorobiphenyl	34883-39-1	PCB	5.22[d]	0.0236	0.82	8	0.0063	0.72	11	38,000	80,449	303,351	13,151[i]
Hexachlorobenzene	118-74-1	HCB	5.73[c]	0.0174	0.73	11				12,400	35,727		28,500[f]
p,p′-DDT	50-29-3	DDT	6.91[c]	0.0161	0.68	12	−0.0013	0.11	49	7,600	23,665	−293,089	25,120[f]

[a] The concentrations of DN in water were not determined so k_1 could not be calculated. BCF_A and $BCF_{No\text{-}G}$ were estimated from the mean k_1 of the other chemicals excluding PCB.
[b] Calculated using Advanced Chemistry Development (ACD/Labs) Software Version 11.02 (1994–2011 ACD/Labs).
[c] From Sabljic et al [13].
[d] Calculated according to Schenker et al[14].
[e] BCF_A and $BCF_{No\text{-}G}$ are based on freely dissolved concentrations in water, while BCF_{LT} is based on bulk concentrations in water.
[f] MITI value from EURAS BCF Gold Standard Database (http://ambit.sourceforge.net/euras/), mean of reported values.
[g] BCF on a wet-weight basis, not normalized to 5% lipid as fish lipid content was not reported.
[h] Non-MITI value from EURAS BCF Gold Standard Database, mean of reported values.
[i] From Fox et al [15].
RSE = relative standard error.

with the results from the multichemical exposures in the present study. The EURAS BCF Gold Standard Database (http://ambit.sourceforge.net/euras/) was used to identify substances with high-quality BCF data. Table 1 lists the test substances, the abbreviations used in the present study, and the BCF from the Gold Standard Database.

The experiments were conducted in 200-L aquaria made of fiberglass. The aquaria were circular, with a central overflow drain. The aquaria were supplied with a continuous flow of charcoal-filtered, aerated tap water. An additional filter removed particles down to 10 μm. Water was supplied to the aquaria at 48 L h^{-1}, giving an exchange rate of 5.8 d^{-1}. The water in the aquaria was aerated. The aquaria were covered with plastic, and a pump outfitted with an activated carbon filter was used to maintain an underpressure in the headspace in order to prevent contamination of the laboratory. The water supply had a temperature of 13°C, and the temperature in the aquaria room was maintained at 13°C. The lighting was controlled by an astronomic clock, providing a day: night cycle matching that in Stockholm at the time of the experiments.

Juvenile rainbow trout were used for the experiment. They were purchased from Näs Fiskodling at the age of 12 months and held in the laboratory's aquaria. At the start of the experiment they were 13 months old and weighed approximately 45 g. The fish were fed 3-mm pellets (EFICO Alpha from BioMar) once per day at a rate of 1% of their body weight. Fecal pellets were vacuumed from the bottom of the aquaria before feeding.

Equilibrium passive dosing was used to generate an aqueous solution with constant concentrations of the test chemicals during the uptake experiment. The method is described in detail elsewhere [7]. It employed a PermSelect PDMSA-1.0 silicone membrane module (MedArray), which consisted of two manifolds connected by 16,128 hollow fibers made of polydimethyl siloxane with an inner diameter of 167 μm. The module was loaded by perfusing it with a methanol solution of the test chemicals. After perfusion, the solution was recovered, diluted with water, and reinjected into the module. This was repeated until a water:methanol volume ratio of 19:1 was achieved. Method-validation experiments indicated that this procedure transferred >99.5% of the test chemicals from the solution to the module [7]. Thereafter, water was pumped through the module, and the chemicals partitioned from the module's materials (polydimethyl siloxane and other) back into the water. The concentrations in water were shown to be independent of the water flow rate [7]. By employing the partitioning principle, concentrations are generated that are below saturation and that can be regulated by varying the quantity of chemical loaded into the unit. For most chemicals the concentrations were shown to be constant over time, but for those with lower K_{OW} values (see Table 1) a gradual decrease was seen over periods of days [7]. For this reason, two different modules were used for the experiment: one for the chemicals with higher K_{OW} (pentachlorobenzene [PENT], 2,5-dichlorobiphenyl [PCB], hexachlorobenzene [HCB], dichlorodiphenyltrichloroethane [DDT]) and one for the chemicals with mostly lower K_{OW} (2,6-diisopropyl-naphthalene [DN], 2,3,4-trichloroanisole [ANI], p-diisopropylbenzene [ISBE], musk xylene [MUSK], chlorpyrifos [CHPY], 4-*n*-nonylphenol [NP], 2,4,6-tri-*tert*-butylphenol [BUFE]). Two modules for the high-K_{OW} chemicals were operated in parallel throughout the experiment, while the module for the low-K_{OW} chemicals was exchanged after 14 d with another module that had been loaded in an identical manner.

Water samples (~100 ml) were collected daily during the uptake experiment and for 4 d after, most of the fish had been transferred to the second aquarium for depuration. Sampling was done by immersing a 100-ml glass bottle in the aquarium close to the outflow. Passive water samplers were also deployed on four occasions during this period: on one occasion, one sampler; on one occasion, three; and on the remaining

occasions, two. Silicone sheets ($40 \times 40 \times 0.25$ mm) were first cleaned by Soxhlet extraction in ethyl acetate for 6 h and dried. For sampling they were hung on a steel wire and submerged in the outflow region of the aquarium for 3 h.

Fish were sampled after 2, 4, 8, 13, and 24 d during the uptake experiment and after 10 h and 1, 2, 4, 10, 16, 30, and 57 d during the elimination experiment. On each occasion, four fish were sampled and killed by severing the spine. The fish were stored at -17°C until analysis. Ethical approval for the experiments was obtained from Stockholms Norra Djurförsöksetiska Nämnd (permit N 31/09).

Analytical methods

A solution of surrogate standards in methanol was prepared. The surrogate standard used for each analyte is given in Supplementary Data, Table S1. A 2-ml portion of the surrogate standard solution was added to the water. The weight of the tube was recorded before and after water sampling. The sample was transferred to a 50-mg ENV+ SPE cartridge. After the cartridge had been dried with nitrogen, the analytes and surrogate standards were eluted with 1 ml of ethyl acetate containing 40 ng of decachlorobiphenyl as a volumetric standard.

Silicone sheets were extracted by sonication in 6 ml ethyl acetate for 30 min. After removal of the silicon sheet and addition of the surrogate standards, the solvent was reduced to 1 ml.

Each fish was homogenized using a Kenwood Multipro food processor. The homogenate was extracted by shaking it with 220 ml *n*-hexane/acetone (5:1) in a 1-L Ehrlenmeyer flask on a KS 501 digital shaker for 2 h. The homogenate was allowed to stand for sedimentation, after which the organic phase was decanted. The extraction procedure was repeated with 100 ml hexane. An aliquot corresponding to 20 g fish was taken from the combined organic phases, reduced to 40 ml, and washed with 30 ml 9% sodium chloride in 1M phosphoric acid. The aqueous phase was reextracted with 10 ml hexane. The combined organic phases were then reduced to 8 ml. An aliquot of 100 μl of the solution was transferred to a preweighed test tube and evaporated under a stream of nitrogen to dryness, after which the fat content was determined [8]. The residue was redissolved in 2 ml hexane containing the internal standards and treated with 4 ml sulfuric acid monohydrate for lipid removal. The organic phase was reduced to 1 ml and analyzed by GC-MS.

Gas chromatographic separation was performed on a CarloErba GC8000 gas chromatograph using a $30\,\text{m} \times 0.25$ mm DB-5MS J&W Scientific column with a 0.25 μm film thickness. Injections were made in the splitless mode (260°C) with the GC oven at 80°C. This was held for 2 min, then raised to 300°C at a rate of 10°C/min. Mass spectrometric determination was made using a Finnigan Voyager low-resolution mass spectrometer in electron impact mode. The chemical quantities in the water samples were normalized to water volume, those in the silicone sheets to silicone mass, and those in the fish homogenates to lipid mass.

RESULTS AND DISCUSSION

Fish health

No signs of adverse health were observed during the experiment. There were two cases of mortality due to fish jumping out of the aquarium into the drain. The lipid content of the whole-fish homogenates averaged 7.6 mass percentage. Lower lipid contents were observed in several fish killed toward the end of the uptake experiment and the beginning of the elimination experiment (see Supplemental Data, Fig. S1). The fish grew continuously. A semilogarithmic plot of lipid mass versus time yielded a lipid growth rate constant of $0.0144 \pm 0.0028\ \text{d}^{-1}$ (see Supplemental Data, Fig. S2).

Concentrations in water

Only ANI, CHPY, and MUSK could be determined in the collected water. Therefore, the passive samplers were used to provide the concentrations in water. The silicone sheets functioned as kinetically limited passive samplers; preliminary experiments had shown that they did not approach equilibrium after a sampling time of 20 h (Supplemental Data, Fig. S3). The water data for the three chemicals determined in the water samples were used to determine deployment-specific sampling rates for each of the passive samplers (see Supplemental Data, Table S2). The average of the sampling rates of the three chemicals, which ranged from 0.021 to 0.038 L water g^{-1} silicone h^{-1}, was then employed to calculate the concentrations of the other chemicals in water from the chemical quantities in the passive sampler.

The concentration for each chemical in water is plotted in Supplemental Data, Figure S4. As measurements were made at only four time points, the experiment was divided into two periods separated by the exchange of the passive dosing unit after 14 d, and the measured concentration was assumed to apply to the intervals defined by this boundary and the time points halfway between the two measurements made during each period.

For three of the four chemicals for which one passive dosing unit was used for the whole experiment, the concentrations varied little (PCB, HCB, DDT). A continuous decrease was seen for the fourth (PENT), indicating that it had gradually been depleted in the passive dosing system. For most of the chemicals for which the passive dosing unit was exchanged, the concentration decreased somewhat during the operation of the first unit, as had been anticipated (ANI, ISBE, MUSK, CHPY). It then increased markedly after the units had been exchanged, a consequence of suboptimal unit-to-unit reproducibility of the passive dosing method. The behavior of the two phenolic compounds was less consistent, suggesting that the concentrations in water may have been less constant or the analytical method less precise. The analytical method did not perform satisfactorily for one compound (DN, no results shown).

Concentrations in fish

The lipid-normalized concentrations measured in the fish during the uptake phase are plotted in Supplemental Data, Figure S4. The variability between fish sampled at a given time point is considerable, with the range for the four samples frequently exceeding a factor of 2. For most compounds the concentrations increased throughout the exposure phase. Notable exceptions were ANI and NP, for which the concentrations decreased during the first half of the experiment. In the case of ANI, the concentration time trend in the fish matched that in the water. This is discussed further below. For NP the time trends did not correspond, which may be related to the greater uncertainty in the concentrations in water (see above). It appeared that ISBE had an anomalously low concentration in the fish killed after 8 d of exposure.

The lipid-normalized concentrations measured in the fish during the depuration phase are given in Supplemental Data, Table S3. The variability in concentrations at a given time point again frequently exceeded a factor of 2. Some compounds (in particular NP, ANI, and ISBE) were below the limit of

quantification in fish sampled in the latter part of the experiment due to rapid elimination kinetics.

Elimination rate constant k_T

As described, the uptake and elimination kinetics were assessed using a one-compartment model with first-order kinetics. The elimination rate constant k_T was calculated from the linear regression of a semilogarithmic plot of the lipid-normalized concentration in fish versus the depuration time. Most of the chemicals showed a linear relationship between ln C and time (as shown for PCB and MUSK in Fig. 2 and for the remaining compounds in Supplemental Data, Fig. S5). This indicates that the one-compartment model with first-order kinetics is appropriate. However, ANI showed a different behavior, with rapid elimination during the first day of the elimination experiment, followed by slow elimination during the remainder (Fig. 2). This indicates that a one-compartment model may not be appropriate for this chemical.

The elimination rate constants are listed in Table 1 together with their relative standard error and the correlation coefficients of the linear regression. A low uncertainty in the estimate of k_T is important as it is directly linked to the uncertainty in the BCF estimate. For most chemicals, the relative standard error of k_T was <15%. The only exception was NP, where the higher uncertainty was due to the comparatively short period over which the depuration could be measured (2 vs 57 d for most chemicals). In regulatory contexts in which the purpose is to establish whether the chemical exceeds a certain BCF threshold, this would not be a problem because rapidly eliminated chemicals have low BCFs and will fall well below the thresholds. Those of the studied chemicals that had very low values of k_T (BUFE, HCB, DDT) also had somewhat higher relative standard errors (11–14%). This is a reflection of the stronger influence that variability between fish has on the confidence interval when the slope is very shallow. Finally, ANI showed a somewhat higher relative standard deviation (13%), which can be attributed to the non-first-order behavior discussed above.

The measured elimination rate constants for BUFE, HCB, and DDT are very close to the rate constant for lipid growth ($0.0144\ d^{-1}$, see above); the 95% confidence interval of the k_T

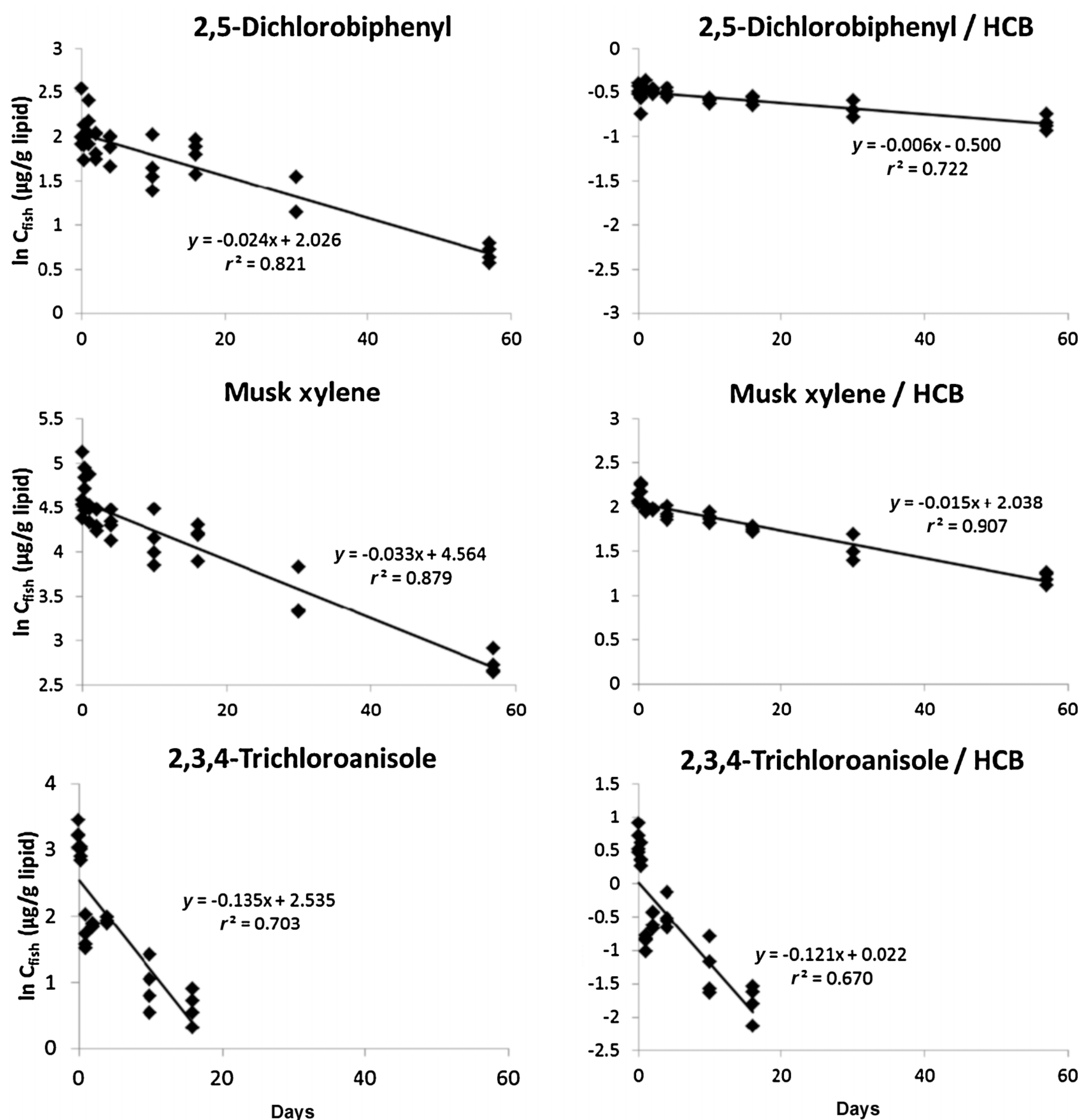

Fig. 2. Semilogarithmic plot of the concentrations of 2,5-dichlorobiphenyl, musk xylene, and 2,3,4-trichloroanisole in fish during the elimination experiment (left-hand side) and of the same data normalized to hexachlorobenzene (HCB, right-hand side). Lines and equations show the linear regressions of the data. The slopes in the figures on the left represent k_T, while those in the figures on the right represent k_2. For each substance, the same scale is used for the *y* axis, to facilitate comparison of the slope of the line and the scatter of the data.

values (mean – 2 × standard error) overlaps with the lipid growth rate constant. This indicates that there is no statistically significant difference between the overall elimination rate constant and the lipid growth rate constant for these chemicals. In such cases, the k_T value (and thereby the BCF) primarily reflects the growth rate of the fish in the particular experiment, not any intrinsic bioaccumulation property of the chemical. The BCFs calculated from such data are unlikely to have relevance for bioaccumulation behavior in the field because growth rates of fish in the field are typically lower than those in the laboratory, and hence BCFs would be higher. The consequence of this is that there is an upper limit for the BCF that can be measured in this kind of laboratory experiment. This limit is determined by the growth rate of the fish, and it can be raised by having slower-growing fish in the experiment (see Fig. 1).

One way to directly address this limitation is to calculate growth-corrected BCF values ($BCF_{No\text{-}G}$). This is done by employing a growth-corrected overall elimination rate constant (i.e., k_2), which is equal to the difference between k_T and k_G (see Eqn. 4)

$$k_2 = k_T - k_G \tag{6}$$

k_2 can be elegantly determined using a contaminant with very slow elimination kinetics as an internal benchmark of growth dilution within the experiment. For such a benchmark, k_T and k_G are essentially the same; hence, k_G can be approximated as the k_T measured for the benchmark. Recalling the equations for the calculation of the rate constants

$$k_T = -\frac{\ln C_{A,t2} - \ln C_{A,t1}}{t_2 - t_1} \tag{7}$$

$$k_G = -\frac{\ln C_{B,t2} - \ln C_{B,t1}}{t_2 - t_1} \tag{8}$$

where C is concentration, t is time, and A and B refer to analyte and benchmark, respectively. Subtracting the two rate constants gives

$$\begin{aligned} k_2 &= k_T - k_G \\ &= -\frac{\left(\ln C_{A,t2} - \ln C_{A,t1}\right) - \left(\ln C_{B,t2} - \ln C_{B,t1}\right)}{t_2 - t_1} \\ &= \frac{\left(\ln\left(C_{A,t2}/C_{B,t2}\right) - \ln\left(C_{A,t1}/C_{B,t1}\right)\right)}{t_2 - t_1} \end{aligned} \tag{9}$$

Hence, the growth-corrected elimination rate constant k_2 can be directly calculated from the ratio of the concentration of the analyte and the benchmark. For a data set with more than two data points, k_2 is obtained from a linear regression of the natural logarithm of this ratio against time.

Working with ratios of concentrations instead of absolute values has several further advantages when evaluating the experimental data. First, the analytical precision when measuring the ratio of concentrations is often greater than the precision when measuring absolute concentrations. Second, the use of ratios can reduce the variability introduced by differences between individual fish. For instance, there may be large differences in the concentrations of a chemical between two fish because of differences in their size or ventilation rate. However, the concentration ratio of two chemicals that were present in the water is likely to be more similar in the two fish.

Another method that has been used to correct for growth dilution is to measure the body burden of the chemical in the fish over time. This has been useful in assessing the elimination kinetics in fish following dietary exposure [9]. The body-burden method requires knowledge of the quantity of chemical in the fish at the beginning of the elimination experiment. In dietary-exposure experiments this can be estimated with some certainty, particularly if the chemicals are delivered via gavage. However, in a bioconcentration experiment in which the fish are exposed via the gills, considerable variation in the fishes' body burden at the end of the uptake experiment/beginning of the elimination experiment is often observed due to interindividual differences in ventilation rate and metabolism. For instance, in the present study, the quotient of the maximum/minimum body burdens in the four fish sampled at the end of the uptake experiment ranged from 2.8 (for NP) to 5.1 (for ANI). This high variation limits the applicability of the body-burden approach in bioconcentration experiments.

To illustrate the internal benchmark approach for correcting for growth dilution, HCB was selected as a growth benchmark and k_2 was calculated for all other chemicals. Figure 2 compares the standard elimination plot (left-hand side) with the growth-corrected elimination plot (right-hand side) for PCB, MUSK, and ANI, while Table 1 lists the k_2 values and their relative standard errors for all chemicals. As expected (see *Theory*), the influence of the growth correction decreases with the magnitude of k_2. For PCB, k_2 is approximately a factor of 3 smaller than k_T and has a correspondingly smaller standard error (0.0063 ± 0.0007). If k_2 had been estimated by taking the difference between k_T for PCB and HCB (i.e., 0.0236 ± 0.0019 to 0.0174 ± 0.0018), the uncertainty would have been larger (e.g., 0.0026 using propagation of error and assuming no covariance). This illustrates the reduction of interindividual uncertainty when concentration ratios are used. If k_2 had been estimated by taking the difference between k_T for PCB and the lipid growth rate constant (i.e., 0.0236 ± 0.0019 to 0.0144 ± 0.0028), as has been the practice in the past, not only would the uncertainty have been larger but the resulting estimate of k_2 would have been different (0.0092). The difference arises because the latter method assumes that one growth dilution correction applies to all fish, whereas the benchmarking method essentially applies an individual specific growth dilution correction to each data point.

This example also illustrates how the calculation of k_2 clearly identifies chemicals that lie outside the range of applicability of the method. The k_2 relative standard error for BUFE exceeds 100%; that is, the estimated rate constant was not significantly different from zero and, hence, was not reliably quantified. In addition to a high relative standard error, DDT has a negative value for the rate constant. Both of these chemicals lie outside the range of applicability of this experiment for the determination of BCF because the rate of chemical elimination from the fish is less than the rate of growth dilution ($k_2 < k_G$).

Uptake rate constant k_1

The uptake rate constant k_1 was estimated for each chemical by fitting the equation for chemical accumulation from the one-compartment model

$$C_{F,t+\Delta t} = C_{F,t} + \left(k_1 C_W - k_T C_{F,t}\right)\Delta t \tag{10}$$

to minimize the sum of the squares of the difference between measured and predicted lipid normalized concentrations in fish during the uptake experiment, whereby C_W varied over time, as shown in Supplemental Data, Figure S4, and k_T was taken from Table 1. This approach allows the variability in C_W, which in some cases exceeded the guidelines in the OECD 305 protocol, to

be taken into consideration in the determination of k_1. The fit of the predicted and measured C_F is illustrated in Supplemental Data, Figure S4, and the values of k_1 are tabulated in Table 1.

The k_1 values were similar for all chemicals except PCB, ranging over a factor of 2.6 between 4,700 and 12,400 L kg^{-1} lipid d^{-1}. No correlation was observed between k_1 and log K_{OW}, and no other relationship between k_1 and physical chemical properties was apparent. For PCB, a much higher k_1 value was observed (38,000 L kg^{-1} $lipid^1$ d^{-1}), and no explanation could be found for this. Because C_W was not available for DN, the k_1 of this chemical could not be determined. For the purpose of estimating the BCF (see below), the k_1 of DN was estimated as the mean of the k_1 for all other chemicals except PCB.

The measured k_1 values were compared with values predicted using two models. The Sijm model [10] predicts k_1 (in L kg^{-1} wet wt d^{-1}) from fish mass (W, in g) according to

$$k_1 = 520\,W^{-0.32} \quad (11)$$

Using the average mass of the fish in the uptake experiment (45 g), this equation gives a k_1 value of 154 L kg^{-1} wet weight d^{-1}. Converting this to a lipid weight basis using the average lipid mass fraction of the fish in the uptake experiment (0.068) yields a k_1 value of 2,300 L kg^{-1} lipid d^{-1}. This is approximately 50% of the lowest value measured in the present study.

The Sijm model was derived from data from fish of two sizes: guppies and rainbow trout. While the rainbow trout in Sijm's data set were of a similar size to the rainbow trout in the present study, measurements were made using isolated perfused gills, not living fish. This may explain the higher k_1 values found in the present study.

The Arnot and Gobas model [11] predicts k_1 (in L kg^{-1} wet wt d^{-1}) from fish mass (W, in kg), K_{OW}, and the dissolved oxygen concentration (C_{OX}, in mg L^{-1}) according to

$$k_1 = \frac{1400}{W^{0.35}(1.85 + 155/K_{OW})C_{OX}} \quad (12)$$

With this model, k_1 is independent of K_{OW} in the K_{OW} range of the chemicals used in the present study. Assuming oxygen saturation in the aquarium of 70% (the range of measured values was 60–75%), the temperature in the aquarium (13°C), and the average mass of the fish killed during the uptake experiment (0.045 kg), one obtains a k_1 value of 295 L kg^{-1} wet weight d^{-1} or, using the conversion to lipid mass above, 4,300 L kg^{-1} lipid d^{-1}. This is slightly lower than the lowest value measured in the present study (4,800 L kg^{-1} lipid d^{-1}). The Arnot and Gobas model is derived from an allometric relationship between oxygen consumption rates and fish mass. The data set used to derive this relationship provides insight into the variability that can be expected for k_1. For a given fish mass the oxygen consumption rate ranged over one to three orders of magnitude, and within the range of fish mass used in the present study the bulk of the oxygen consumption rate data were above the regression line that was employed in the Arnot and Gobas model [12]. Thus, the k_1 values measured in the present study would appear to be consistent with the empirical basis for the Arnot and Gobas model.

Bioconcentration factor

The value of apparent bioconcentration factor (BCF_A) was calculated by dividing k_1 by k_T, and the value of growth corrected bioconcentration factor ($BCF_{No\text{-}G}$) was calculated by dividing k_1 by k_2. Both BCFs were converted to a whole-tissue basis assuming a 5% lipid content (see Table 1). The differences between BCF_A and $BCF_{No\text{-}G}$ vary from 5% for ISBE up to a factor of 4 for PENT and PCB. The influence of growth on BCF_A increased with the magnitude of BCF, as expected from Figure 1. For CHPY and DN, which had a $BCF_{No\text{-}G}$ (3,583 and 5,550) that was close to the regulatory thresholds of 2,000 and 5,000, the two BCF measures differed by 16 and 20%, respectively. Thus, growth dilution during the BCF experiment can be important when assessing whether a chemical exceeds these thresholds.

Growth dilution has a much greater impact for chemicals with higher BCF values (Fig. 1). Although accurate measures of BCF values >10,000 are not required for testing exceedances of regulatory thresholds, they may be required for exposure assessment. The present study shows that in such cases it is important to assess whether the BCF values have been growth-corrected and whether they were within the domain of applicability of the experiment. The impact of growth dilution can be reduced, and thereby the domain of applicability of the experiment expanded, by reducing the growth rate of the fish in the experiment. However, this should not be done at the cost of influencing the health status of the fish. As illustrated in the present study, the use of an internal benchmark for growth dilution can reduce the uncertainty in the correction for growth dilution, which should contribute to better BCF estimates. In selecting the internal benchmark one should remember that the benchmark chemical should be primarily sequestered in the same tissues as the chemical of interest. The chemical HCB is primarily stored in fish lipid and is a suitable benchmark for neutral lipophilic chemicals. For chemicals that partition primarily into other tissues (e.g., PFOS), another benchmark should be chosen.

Table 1 also lists the best available estimates of BCF from the literature. These were largely culled from the EURAS bioconcentration factor Gold Standard Database. In Figure 3, the measured BCF_A and the literature BCF (BCF_{LT}) are plotted against each other. For CHPY and NP they agreed within a factor of 2, while for DN and MUSK they differed by just 3 and 35%, respectively. The agreement was poorer for ISBE, for which BCF_A was three times lower than BCF_{LT}. The agreement was poorest for ANI (> factor of 4), which might be due to the application of a kinetic model to estimate BCF_A, while BCF_{LT}

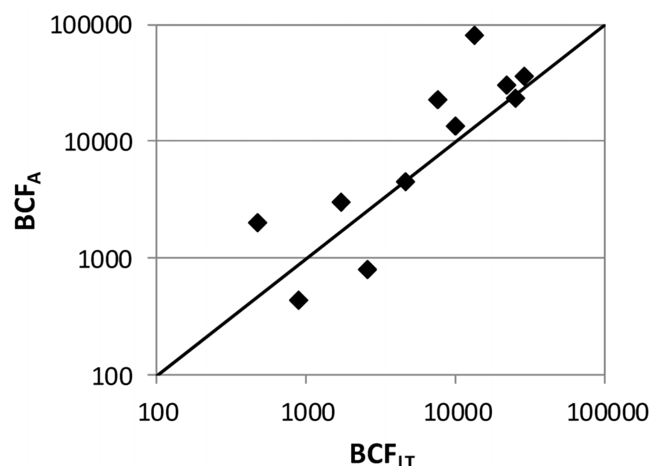

Fig. 3. Plot of the apparent bioconcentration factor measured in the present study (BCF_A) versus the BCF values selected from the literature (BCF_{LT}). The line of 1:1 agreement between the two values is also shown.

was based on the quotient of the concentrations in fish and water at steady state. As noted above, the use of the kinetic model for ANI was problematic as it did not show first-order kinetics. The BCF_A for ANI was also calculated from the concentrations in fish and water after 13 d when the fish was close to steady state (see Supplemental Data, Fig. S4), and the value obtained (748 L kg wet wt^{-1}) was in much better agreement with BCF_{LT} (470 L kg wet wt^{-1}). A comparison of BCF_A and BCF_{LT} for the other compounds, which had BCF_A >20,000, yields insight only into the growth dilution during the respective experiments as the BCF_A is almost entirely controlled by the growth rate for these compounds.

The differences between BCF_A and BCF_{LT} can be compared with the variability of BCF_{LT}. For instance, the Gold Standard Database contains two BCF values for CHPY. Both were taken from the MITI database and determined from the same species and with the same protocol, but the two BCF values differed by a factor of 5.8 (493 vs 2,880). When this variability is taken into consideration, together with other differences between the present study and the literature studies (e.g., different fish species, normalization to bulk water concentration in the literature studies vs freely dissolved concentration in the present study), the BCF_A values from the present study are largely consistent with those in the literature. These results are encouraging, and further testing comparing single-chemical exposures and multiple chemical exposures of the same fish using the same experimental and analytical protocols in the same laboratory is suggested to further explore this issue.

No evidence was found to suggest that the measurement of several chemicals simultaneously led to incorrect results. However, caution should be exercised when applying the multiple-chemical approach to chemicals for which metabolism is expected to be the dominant elimination mechanism. If a second chemical in the mixture either inhibits or induces enzymes involved in the metabolism of the test chemical, errors in BCF determination may result. This can add another level of uncertainty to that already contributed by the possible influence of the test chemical itself on its own metabolic elimination (the fact that BCF laboratory studies are typically performed at exposure levels far above those expected in the environment can result in nonrepresentative inhibition/induction of enzymes in the laboratory experiment). One must also be wary of the potential increased risk of toxicological effects posed by exposure to multiple chemicals. The OECD 305 guideline recommends selecting the highest exposure concentration to be approximately 1% of the acute asymptotic LC50 value [1]. It is advisable to use the lowest exposure levels within the constraints posed by the analytical methodology and the desired precision of the BCF determination.

SUPPLEMENTAL DATA

Table S1. Substances used and their corresponding internal standards and suppliers.

Table S2. Sampling rates of the passive samplers.

Table S3. Lipid normalized concentrations of the test chemicals in the fish sacrificed during the depuration phase.

Figure S1. Lipid content of the fish (%) during the experiment.

Figure S2. Lipid mass of the fish during the experiment.

Figure S3. Concentrations of test chemicals in silicone sheets versus the time that the sheets were deployed in the aquarium.

Figure S4. Measured concentrations in water, measured concentrations in fish, and modeled concentrations in fish during the uptake experiment.

Figure S5. Semilogarithmic plot of the concentrations in fish during the elimination experiment. (1,052 KB DOC).

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