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Bioconcentration of cedarwood oil constituents in rainbow trout†

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Cedarwood oil is an essential oil used as a fragrance material and insect repellent. Its main constituents are sesquiterpenes which are potentially bioaccumulative according to the REACH screening criteria. Cedarwood oil is a complex mixture of hydrophobic and volatile organic chemicals. The volatility and limited water solubility of its constituents are a challenge for standard bioconcentration factor (BCF) test methods using aqueous exposure. We used an abbreviated dietary exposure *in vivo* testing protocol with internal benchmark substances as "internal standards" to derive the BCF of cedarwood oil constituents using rainbow trout (*Oncorhynchus mykiss*). Internal benchmarking proved to be a useful tool to control for inter-individual variability, enabling us to calculate the BCF for all major cedarwood oil constituents as a mixture. We found that the BCF of two out of six analysed cedarwood oil constituents exceed a BCF of 5000 and two others exceed a BCF of 2000 (90% confidence level) even though we found evidence for biotransformation for individual constituents. The results of this study indicate that more work is warranted to study the bioaccumulation of essential oils and highlights the utility of internal benchmarking in *in vivo* dietary exposure BCF tests to increase robustness and allow for the BCF measurement of complex mixtures.

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Environmental significance

Our paper reports the simultaneous measurement of the depuration rate constants of 7 volatile and hydrophobic cedarwood oil constituents using *in vivo* dietary exposure with internal benchmarking. The results of our study showed that most sesquiterpene constituents in cedarwood oil are bioaccumulative or very bioaccumulative in rainbow trout. The use of internal benchmarking allowed us to significantly reduce the inter-individual variability of the measured concentrations. This meant that we could use an abbreviated dietary exposure set up and test the constituents as a mixture while maintaining a good statistical robustness. Moreover, using threshold benchmarking and reference compounds for gill elimination, we were able to estimate the biotransformation rate constants for the individual constituents and determine different elimination pathways from the fish. Our findings provide novel insights into the bioaccumulation behaviour of sesquiterpenes and explore internal benchmarking for the investigation of elimination pathways and the assessment of volatile, hydrophobic mixtures that cannot easily be assessed in a standard OECD 305 BCF test. We believe that the results of this study are of interest to academia, regulators, and industry both from a methodological point of view – as a potential method to determine the BCF of UVCBs – as well as from an environmental chemical point of view.

1. Background

Essential oils are extracts from plants with a variety of uses in consumer and industrial products.[1,2] As plant extracts, essential oils are complex mixtures with variable compositions based on factors that include where the plants were grown and how the oil was extracted.[3] One commonly used essential oil is cedarwood oil,[4] which is obtained from the Cupressaceae family that includes cedar, juniper and cypress.[5]

Cedarwood oil is used as a fragrance ingredient in personal care and household products.[4] It is also popular within the wellness and alternative remedies culture, where it has been attributed a variety of health benefits, ranging from prevention of hair loss to the treatment of diabetes.[6] In controlled scientific studies, cedarwood oil has been shown to repel insects,[7] to have

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† Electronic supplementary information (ESI) available: (1) Method for deriving the bioconcentration factor. (2) Method for estimating the uptake rate and assessing its uncertainty. (3) Details on the internal benchmarking approach; incl. conservative benchmarking, threshold benchmarking and the calculation of standard error and confidence intervals. (4) Information on target analytes, cedarwood oil composition (pure oil), constituent patterns found in fish. (5) Details on measured concentrations per samples. (6) Fish weight. (7) Depuration kinetics for target analytes and benchmark substances. (8) Detailed method for k_M prediction. See DOI: 10.1039/d1em00009h

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antimicrobial properties,[8–10] to reduce obesity in rats,[11,12] and to have anxiolytic effects in mice.[13] Due to its attributed properties, cedarwood oil is used in a variety of personal care products as well as in bio-pesticides, insect-repellents and alternative medicinal products.[4,14]

The composition of commercially available cedarwood oils varies depending on which Cupressaceae species it is extracted from. Himachalenes are the primary components of cedar-based cedarwood oils,[15] whereas juniper-, and cypress -based cedarwood oils contain high levels of sesquiterpenes such as cedrene and thujopsene, and sesquiterpene alcohols like cedrol and widdrol.[3,16] Sesquiterpenes and sesquiterpene alcohols are branched hydrocarbons, many with $\log K_{OW}$ above 4.5 and molecular weight below 600 Da (Table S1†), which means they would be flagged as potentially bioaccumulative according to the European Union Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH) screening criteria.[17] The bioaccumulation potential prompted work to test the biodegradability of cedarwood oil constituents[18] which indicated that none of the major cedarwood oil constituents are persistent in an Organisation for Economic Co-operation and Development (OECD) 301F manometric respirometry test for ready biodegradability.

Under REACH, cedarwood oil and other essential oils are classified as substances of unknown or variable composition, complex reaction products or biological materials (UVCB). UVCBs present a challenge to the standard regulatory assessment of the bioaccumulation potential (B) of chemical products, because the assessment procedures employ tests designed for individual chemicals rather than a mixture of substances.[19] Regulators, academia and industry have together called for the development of chemical risk assessment methods specifically for UVCBs.[20]

Furthermore, measuring the bioconcentration factor (BCF) of volatile, hydrophobic substances such as the constituents of cedarwood oil is challenging, as stable exposure concentrations in water cannot be maintained in a standard flow-through experiment. The OECD 305 guideline for the *in vivo* determination of BCF in fish provides the option to determine the BCF using a measured growth-corrected depuration constant (k_{TG}) from dietary exposure and an estimated uptake rate (k_1).[19] However, this approach has been criticised because k_1 estimates can vary widely depending on the model that is selected.[21] Furthermore, dietary exposure and, particularly, abbreviated single dietary exposure studies, suffer from high inter-individual variability in exposure concentrations due to *e.g.* differences in feeding behaviour and success of the test animals.

One way to control for inter-individual variability is the use of "internal benchmarking". Benchmarking in BCF experiments is similar to the use of so-called internal standards in analytical chemistry: well-characterised benchmark substances are used alongside the target analytes throughout the experiment. Concentrations of the target analytes are then measured relative to the concentrations of these well-characterized standards with known behaviours in the test system, *i.e.*, the depuration rate of a target analyte (change in concentration (dC) over time (dt)) is measured relative to the change of the concentration of the benchmark substance (dC_{BM}) over time:

$$\frac{dC}{dt} \Rightarrow \frac{d\left(\frac{C}{C_{BM}}\right)}{dt} \quad (1)$$

Chen *et al.*[22] demonstrated that internal benchmarking with a conservative substance can reduce the inter-individual variability of dietary exposure-based k_T measurements. Another way of using the benchmark concept is to benchmark against a substance with a known BCF close to the bioaccumulative (B) (BCF > 2000) and/or very bioaccumulative (vB) (BCF > 5000) regulatory thresholds.[23] These so-called "threshold benchmark" substances can be used to directly compare the depuration rate of a target analyte to the depuration rate of a known B or vB compound. Assuming that the uptake rates for target analytes and benchmark substances are the same,[19] threshold benchmarking thereby provides a method to assess whether a target analyte meets the B or vB criterion based solely on the benchmarked depuration rate measured in the *in vivo* test, without the need to apply a model to estimate k_1.

Considering the widespread use of cedarwood oil, the potential for emissions into the environment through *e.g.* volatilisation, waste-water, and discharged produced water,[24] as well as detection of α-cedrene in wild-caught fish in the Great Lakes region of North America,[25] it is important to understand the bioaccumulation potential of its constituents. In the presented study, we measured depuration rate constants (k_T) and derived BCFs of constituents in a cedarwood oil in order to gain more understanding of the potential bioaccumulation of this widely used essential oil and to explore the use of threshold benchmarking in abbreviated dietary exposure studies.

2. Materials and methods

2.1 Selection of target analytes and benchmark substances

Virginian cedarwood oil (batch number AS00254371) was provided by Givaudan UK Ltd. Characterisation of the oil composition was performed by Givaudan UK Ltd and was consistent with our own analysis using gas-chromatography coupled with single mass-spectrometry (GC-MS) measurements (in full-scan) (Table S2†) (details on the GC-MS method are provided below). Analytical standards were purchased for all cedarwood oil constituents that contributed >1% of the mixture mass apart from widdrol, which co-eluted with cedrol in our targeted analysis and was therefore reported as a cedrol/widdrol sum parameter. The resulting target analytes were α-cedrene, β-cedrene, cedrol/widdrol, cuparene, α-funebrene, and thujopsene (Table S1†).

Benchmark substances were chosen based on the following criteria: (a) availability of high-quality BCF literature data (applies to both conservative and threshold benchmarks), (b) conservative benchmark substances should not be biotransformed during the depuration phase, (c) threshold benchmark substances should have a BCF of close to 2000 (B) or 5000

(vB), (d) all benchmark substances need to be analysable using GC-MS.

By applying these criteria, pentachlorobenzene (PeCB) and hexachlorobenzene (HCB) were selected as vB threshold and conservative benchmarks, respectively. Additionally, trichlorobenzene (TrCB) was added as a quality control reference substance (for literature/model comparison) for low BCF substances (it was not used as a benchmark substance). D4-Acetyl-cedrene (D4-AC) and ^{13}C-hexachlorobenzene (^{13}C-HCB) were used as isotope labelled surrogate standards. 2,2′,5,6′-Tetrachlorobiphenyl (PCB53) was used as a volumetric standard. All native analytical standards were purchased from Sigma Aldrich, all isotope-labelled standards were purchased from Cambridge Isotope Laboratories. A full list of the target analytes, benchmarks and isotope-labelled standards, including structure, selected physical–chemical properties and analytical parameters are provided in Table S1 in the ESI.† Details on the benchmark methods are presented in the ESI Section 3.†

2.2 *In vivo* experiment

The rainbow trout (*Oncorhynchus mykiss*) used in this study were purchased from Vilstena and Näs fish farms, Sweden and were kept in accordance with the Swedish National Agriculture Board's guidance on research animals (Statens Jordbruksverks Föreskrifter och Allmänna Räd om Försöksdjur, SJVFS 2015:24) and in accordance with the OECD 305 test guideline. The fish were monitored daily for signs of injuries or stress throughout the experiment. No elevated stress levels were observed.

Ethical approval for the experiments was obtained from Stockholms Djurförsöksetiska Nämnd (permit 16938/17).

The *in vivo* experiment was conducted following an eight-week acclimatisation period using the method published by Chen *et al.*[22] In brief, 49 adult rainbow trout (mean weight 95.6 ± 21.1 g) were held in a 350 L aquarium. Fifteen adult rainbow trout were kept in a separate 250 L aquarium as control group. The aquaria were equipped with a pump to aerate the water (flow: 1.5 L min^{-1}) and a filter to remove suspended particulate matter. The water exchange rate in the exposure and control aquaria was 6.5 d^{-1} and 5.8 d^{-1}, respectively. The oxygen concentration was ≥7 mg L^{-1} in both aquaria for the entire experiment. Both the room and the water were held at a constant temperature of 10 °C. The light schedule was 16 (light): 8 h (dark). Faeces residues were siphoned off and the filters of the pumps were changed daily to clean the tank.

The fish in the exposure group were exposed using feed contaminated with 33 μl g^{-1} cedarwood oil as well as 72 μg g^{-1} TrCB, 76 μg g^{-1} PeCB, and 60 μg g^{-1} HCB as benchmark substances during a single feeding exposure event of 0.8% of their body weight. The exposure doses were well below the effect concentrations for the substances.[26] After the initial exposure, both the exposure group and the control group were fed clean food daily at 0.7% of their body weight. Six to eight fish from the exposure group and two fish from the control group were sacrificed on days 1, 2, 4, 7, 14, 21, and 28. The weight of each fish was recorded and the whole fish stored at −20 °C until analysis.

2.3 Sample preparation and analysis

Sample preparation and analysis were conducted according to Chen *et al.*[27] In brief, 5 whole rainbow trout from each sampling day were homogenized individually and 5 g subsamples of each were spiked with surrogate standards (listed above). The samples were extracted with 10 ml acetonitrile using ultrasonication (15 min). The supernatant was decanted into an 80 ml centrifuge tube. The extraction was repeated three times. The four extracts were combined and 25 ml Milli-Q water, 0.5 ml 0.9% sodium chloride solution and 5 ml *n*-hexane were added. This mixture was inverted for 5 minutes and centrifuged for 3 minutes at 3000 rpm (equal to 1006 g). Following centrifugation, the hexane phase was collected in a pre-cleaned 10 ml glass purge tube with a pre-cleaned magnetic stir bar. Subsequently, the analytes were transferred to a SPE column using a purpose-built purge and trap system operated at 70 °C. PCB53 was added as a volumetric standard to control for differences in solvent volume prior to analysis.

Instrumental analysis was performed using a Trace 1300 Series gas chromatograph (GC) (Thermo Scientific) equipped with a TG-5SIL MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film) and helium (constant flow 1 ml min^{-1}) as the carrier gas. A programmed temperature vaporizing (PTV) injector (in splitless mode) and an AI 1310 Autosampler (Thermo Scientific) were used for injection. A single quadrupole ISQ mass spectrophotometer (MS) (Thermo Scientific) was used for detection and quantification of the analytes. The MS was operated in electron ionization (EI) mode (70 eV), and selective ion monitoring (SIM) was used to scan for ions of target compounds with a dwelling time of 0.01 s each. The internal standard method was employed to quantify the target chemicals in the samples. Target ions and used surrogate standards are presented in Table S1.†

2.4 Data processing

Peak identification and integration were done using Thermo Scientific Xcalibur Quantitative Analysis (3.1.66.10) using external calibration. Further data processing was performed using RStudio (version 1.1.456) and Microsoft Excel 2016.

The BCF was calculated kinetically as the estimated uptake rate constant (k_1) divided by the measured total elimination rate constant (k_T) determined in the *in vivo* test. To control for inter-individual variability in the target analyte concentrations among the individual fish (due to differences, for example, in feeding behaviour and growth dilution), the benchmarked total depuration rate constant (k_{TG}) and the benchmarked BCF (BCF_{BM}), respectively, were calculated from the benchmarked concentrations of the target analytes in the individual fish. Concentrations below the LOQ were treated as 0. Details on the methods for calculating the BCF, the estimation of k_1 and the internal benchmarking methods are presented in the ESI Sections 1 (BCF), 2 (k_1), and 3 (benchmarking).†

Furthermore, threshold benchmarking with PeCB as a vB threshold benchmark substance was used to compare the elimination rates for the target analytes to the elimination rate of a substance with a known BCF. Analogous to the conservative benchmarking approach, in threshold benchmarking the

depuration rate constants of the target analytes were calculated from the concentrations of the target analytes benchmarked to concentrations of PeCB in the individual fish. A detailed description of the conservative and threshold benchmarking is provided in the ESI Section 3.† The k_1 for the experiment was estimated based on the model proposed by Arnot and Gobas[28] (the calculations are shown in the ESI Section 2†). The OECD 305 recommends the Arnot and Gobas model[28] as one of the suitable methods to estimate k_1. In previous studies, Chen *et al.*[22] also found that the Arnot and Gobas model results in a good agreement of derived BCFs for neutral hydrophobic substances with respective BCFs measured in standard regulatory test set ups reported in the literature. All substances considered in this study have a $K_{OW} > 1000$ (Table S1†), and modelled k_1 is insensitive to chemical properties for such substances.[22] Thus, a constant k_1 of 120 L kg^{-1} d^{-1} based on the median fish weight (95.6 g) was used in the BCF calculations for all substances.

2.5 QA/QC

The abbreviated test setup with a single exposure event does not allow for the robust measurement of a dietary uptake rate. To confirm that the fish had been exposed to the analytes, rapid screening of aliquots from three fish sampled on day 1 was conducted (full scan, m/z 50–350). Five exposed fish per time point were analysed. One fish from each batch of five was analysed in duplicate. Two control group fish per time point (fish tissue blank) and a method blank without fish tissue per batch were analysed as blanks. Spike recoveries using native standards were conducted with and without matrix in triplicate for all target analytes. Recoveries of the surrogate standards D4-AC and ^{13}C-HCB were determined for each sample. The limit of detection (LOD) was calculated as three times the standard deviation of the concentration in the control group fish (fish tissue blank). The limit of quantification (LOQ) was calculated as 10 times the standard deviation of the concentration in the control group fish (fish tissue blank).

3. Results

3.1 QA/QC

All analytes were detected in fish sampled on day 1, confirming successful exposure (Fig. S1†). Average concentrations in the control group (fish tissue blank) ranged from 3.5 ± 0.57 (standard deviation) ng g^{-1} wet weight (ww) for HCB to 59 ± 33 ng g^{-1} ww for cuparene resulting in an LOD ranging from 1.7 ng g^{-1} ww for HCB to 100 ng g^{-1} ww for cuparene and an LOQ ranging from 5.7 ng g^{-1} ww for HCB to 332 ng g^{-1} ww for cuparene, respectively. The detected concentrations in the control group were between 2 and 4 orders of magnitude below the concentrations measured in the exposure group. The concentrations in the method blanks without fish tissue were similar to the concentrations in the fish tissue blanks. The average recoveries were 99 ± 17% for ^{13}C-HCB and 105 ± 30% for D4-AC. The average overall relative recoveries (after correction with surrogate standards) for the target analytes ranged from 84% ± 8% for cedrol to 113% ± 6% for α-cedrene without matrix and 75% ± 11% for cedrol to 92% ± 9% for α-cedrene with matrix. The data were blank and recovery corrected. Information on method optimization has previously been reported by Chen *et al.*[27]

The fish growth was negligible (<1% increase in average fish weight) for the duration of the experiment (Fig. S2 and Table S3†). HCB concentrations did not follow any significant trend throughout the depuration phase (Fig. S3†). The lack of elimination within the 28 days test was consistent with reported BCFs[29] of >20 000, confirming applicability of HCB as a conservative benchmark. Similarly, the median BCF of 4850 L kg^{-1} ww for PeCB was in good agreement with literature values (ranging from 5130–6920 L kg^{-1} ww).[29] The BCF around 5000 L kg^{-1} ww confirmed its applicability as a vB threshold benchmark.

3.2 Cedarwood oil constituent pattern

α-Cedrene, β-cedrene, and thujopsene were detected in 100% of the samples from the exposure group at concentrations well above the LOQ. Consistent with the constituent pattern in pure cedarwood oil, α-cedrene was the dominant compound in all exposed fish. The contribution was not uniform, but increased over the course of the experiment, with over 50% contribution in fish sampled on days 14 to 28 (Fig. S4†). Thujopsene concentrations in fish were next highest among cedarwood oil constituents, contributing around 30% of the contaminant load for the duration of the experiment (Fig. S4†). Cedrol/widdrol made the third highest contribution in the exposed fish sampled on days 1 to 4, whereas its contribution to the contaminant load dropped to <10% in fish sampled at day 8 and 14 with <50% quantifiable concentrations in samples from day 14. After day 14 cedrol/widdrol was only detected in a few samples at concentrations close to the detection limit (contribution <1%) (Fig. S4 and Table S3†). The remaining target analytes detected in exposed fish had contributions of <10%. α-Funebrene was quantifiable in ≥50% of fish from the exposure group throughout the experiment (Table S3†) but had the lowest contributions to the contaminant load with <1% (Fig. S4†).

3.3 Depuration rate and bioconcentration factor using conservative benchmarking with HCB

Conservative benchmarking with HCB reduced the inter-individual variability of the detected concentrations and derived BCFs considerably (Fig. 1), reducing the standard error of the non-benchmarked depuration rate constant (k_T) by a factor of 1.3 (α-funebrene) to a 3.3 (α-cedrene) (Table S4†). Without conservative benchmarking, the BCF for four out of the six targeted cedarwood constituents could not be quantified due to the high variability in the measured concentrations that resulted in depuration rates not statistically different from zero. Using conservative benchmarking, BCFs could be estimated for all target analytes (Table 1 and Fig. 1).

Cedrol/widdrol was eliminated most rapidly with a benchmarked depuration rate constant (k_{TG}) of 0.23 ± 0.036 d^{-1} while α-cedrene had the slowest elimination with a k_{TG} of 0.013 ± 0.007 d^{-1} (Table S4† and Fig. 1).

Consequently, the derived benchmarked BCF (BCF_{BM}) was lowest for cedrol/widdrol with a median of 525 L kg^{-1} ww

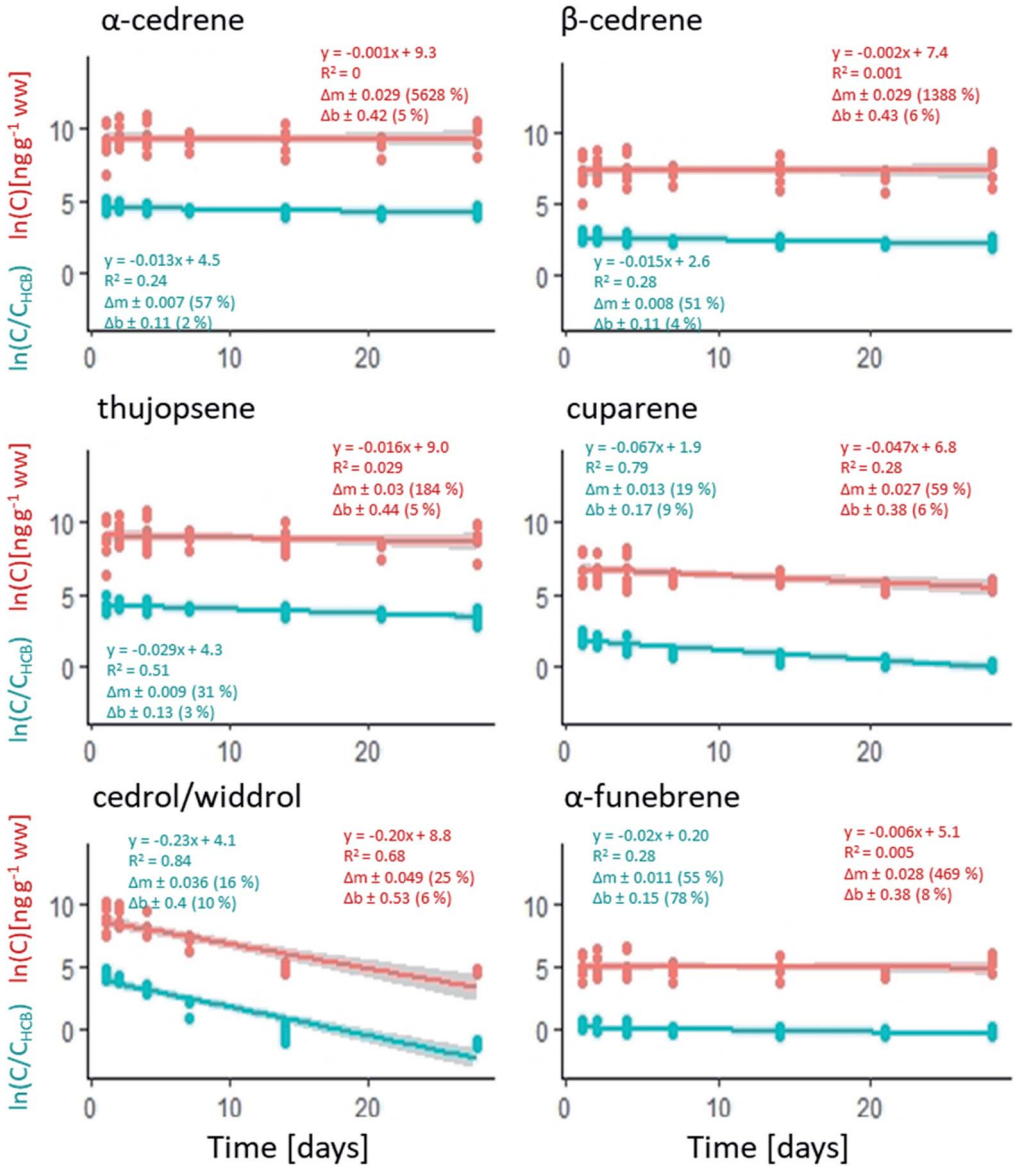

Fig. 1 Depuration kinetics ($y = mx + b$) for the main cedarwood oil constituents plotted as the natural logarithm (ln) of the measured target analyte concentrations [ng g^{-1} ww] (orange) and the ln target analyte concentrations benchmarked with HCB (blue) against the time [days].

(430 L kg^{-1} ww to 720 L kg^{-1} ww) and highest for α-cedrene with a median of 9199 L kg^{-1} ww (6900 L kg^{-1} ww to 23 000 L kg^{-1} ww) (Table 1). Apart from cedrol/widdrol and cuparene, all analysed cedarwood oil constituents met the B screening criterion (>2000) at a 90% confidence level (Table 1). Both α-cedrene and β-cedrene additionally met the vB criterion (>5000) at the 90% confidence level. α-Funebrene had a median BCF_{BM} above the vB threshold, while at the 5th percentile the BCF_{BM} was slightly below the regulatory threshold (Table 1). Thujopsene had a median BCF_{BM} of 4171, which is just below the regulatory threshold for vB (Table 1). Cuparene had a median BCF_{BM} of just below the B threshold at 1781 (Table 1).

3.4 Results from threshold benchmarking with PeCB

Using PeCB as a benchmark reduced the inter-individual variability of the results to a similar extent as when benchmarking with HCB (Fig. S5† and Table 2). Threshold benchmarking the target analyte concentrations resulted in a negative k_{TG} (*i.e.* slower elimination than the benchmark substance) for α-cedrene and β-cedrene at the 90% confidence level. The k_{TG} of α-funebrene was negative, while slightly above 0 (0.005) at the 5th percentile. Thujopsene had a k_{TG} close to 0 (0.004) with a negative k_{TG} at the 95th percentile.

4. Discussion

4.1. Internal benchmarking in *in vivo* dietary exposure BCF testing

The results of this study highlight the utility of internal benchmarking to control for inter-individual variability in *in vivo* BCF experiments. Without benchmarking, the 95th percentile of BCFs could not be determined for the majority of

Table 1 Median, 5th and 95th percentile of BCF [L kg^{-1} ww], BCF_{BM} [L kg^{-1} ww] (benchmarked with HCB), and log K_{OW} for the main cedarwood oil components and BMs

	BCF			BCF_{BM}				
Target	5th	Median	95th	5th	Median	95th	Literature BCF	log K_{OW}[b]
α-Cedrene	4600	138 000	>138 000	6900	9199	23 000	n/a	5.74
β-Cedrene	4400	69 000	>69 000	6000	8203	20 000	n/a	5.82
Thujopsene	3000	8600	>8600	3600	4171	6900	n/a	6.12
Cuparene	1900	2900	6900	1500	1781	2600	n/a	6.19
Cedrol/Widdrol	570	710	940	430	525	720	n/a	4.38/4.84
α-Funebrene	4100	23 000	>23 000	4400	6115	15 000	n/a	5.75
BMs								
TrCB	610	800	1200	520	650	870	430–1700[a]	4.02
PeCB	3500	12 000	>12 000	3800	4850	9900	5130–6920[a]	5.17
HCB	12 000	>12 000	>12 000	n.a.	n.a.	n.a.	27 000–30 000[a]	5.73

[a] Cefic "gold standard" BCF database.[28] [b] Predicted by EPISuite v.4.11.

Table 2 k_T and $k_{TG} \pm 1.96 \times$ SE [d^{-1}] (5th and 95th percentile), threshold benchmarked with PeCB for the main cedarwood oil components and BMs. Substances that meet the vB criterion on a 90% confidence level based on the threshold benchmarking have been marked in bold

Name	k_T	k_{TG}
α-Cedrene	0.001 ± 0.029	**−0.012 ± 0.01**
β-Cedrene	0.002 ± 0.029	**−0.01 ± 0.009**
Thujopsene	0.016 ± 0.03	0.004 ± 0.011
Cuparene	0.047 ± 0.027	0.044 ± 0.012
Cedrol	0.195 ± 0.049	0.203 ± 0.036
α-Funebrene	0.006 ± 0.028	−0.004 ± 0.009
BMs		
TrCB	0.173 ± 0.053	0.15 ± 0.024
PeCB	0.012 ± 0.027	n/a
HCB	−0.013 ± 0.024	**−0.025 ± 0.011**

target analytes in this study, whereas internal benchmarked data yielded 95th percentile BCF estimates that were relatively well constrained, even for constituents that were above the vB threshold.

The use of both conservative and threshold benchmark substances reduced the confidence interval of the benchmarked results compared to the non-benchmarked results. This means the co-variate inter-individual variability based on *e.g.* differences in feeding behaviour and analytical errors in the experiment was greater than independent variability based on *e.g.* differences in elimination pathways for different chemicals. The benchmarking also corrects for inherent sources of variability in the elimination rate constant (and hence BCF), such as fish size and lipid content.

Moreover, the bioaccumulation hazard classification of the chemicals was consistent between benchmarked BCFs derived with conservative benchmarking and relative depuration rate constants using PeCB as a vB threshold benchmark substance (Tables 1 and 2). This indicated that threshold benchmarking with PeCB could be used to determine whether the constituents are above or below the vB threshold based on the depuration rate constants alone. This approach eliminates the problems associated with variability between models for estimating k_1 by effectively replacing the k_1 model with the assumption that k_1 is equal for PeCB and the test substance. It is important to note that the assumption that k_1 is equal for benchmark and test substances may not apply to all substances, *e.g.* very large molecules, charged molecules, or molecules with a $K_{OW} < 1000$.

In this experiment, the similarity between results with estimated k_1 (BCFs derived from conservative benchmarking) and results based on the depuration rate constant alone (threshold benchmarking) indicated that the chosen estimation method for k_1 resulted in accurate k_1 estimates for the target analytes in this experiment. This conclusion was further supported by the good agreement of the derived BCF for the benchmark substances with previously published BCFs.

4.1.1. Bioaccumulation potential of cedarwood oil. The results from the benchmarked *in vivo* tests showed that four of the six main constituents of Virginian cedarwood oil are bioaccumulative or very bioaccumulative in this test set up. Due to the high bioaccumulation potential of both α-cedrene and β-cedrene, as well as thujopsene in this test set up, it can be inferred that juniper- and cypress-based cedarwood oils, in general, are likely to be tested as containing very bioaccumulative constituents because, despite the differences in specific composition, the main constituents of these oils are cedrene, cedrol and thujopsene.[3,16]

4.1.2 Potential sources for systematic errors that the applied benchmarking approach does not correct. The presented test method differs from the OECD305 protocol and it has to be considered that some of these deviations could lead to an overestimation of the derived BCFs that will not be corrected by internal benchmarking. Firstly, the concentrations tested in this study were considerably higher than prescribed in OECD 305 and only one concentration was tested. The high concentrations could, theoretically, lead to enzyme saturation and thereby reduce the biotransformation potential for the test substances. Secondly, the presented method uses dietary exposure. Consequently, potential biotransformation at the gills is not taken into consideration.

Potential enzyme saturation as a source of error for BCF measurements has been discussed in the guidance document to OECD 305.[19] The guidance document reported that no significant differences between BCFs obtained from standard OECD 305 tests using aqueous exposure with low and high test concentrations, respectively were observed. It was concluded that "the differences between BCF_{best} estimates from the higher and lower concentrations mainly reflect apparently random influences." rather than potential suppression of elimination of the test chemicals due to enzyme saturation (OECD, 2016, page 15, paragraph 61).[19] However, the guidance document does not rule out that there might be certain substances for which the test concentration can have an impact on the measured BCF.[19] Burden *et al.*[30] investigated the concentration dependence of 236 BCFs from aqueous exposure collected in the Cefic "gold-standard" bioconcentration database.[29] They found that concentration dependence occurred mostly for substances with BCFs < 100, whereas no concentration dependence was observed for substances with BCFs > 1000. These findings were consistent with the findings documented in the guidance document to the OECD TG 305,[19] as well as reported concentration dependence for BCFs of pharmaceuticals.[31]

Regarding potential biotransformation at the gills, the OECD 305 guidance document argues that the *in vivo* accumulation potential of a chemical is independent of the uptake route.[19,32] Other authors have argued that biotransformation during the uptake can significantly impact the bioconcentration potential of a chemical.[33–35] Lack of gill biotransformation could lead to a potential overestimation of BCFs.[33] At the same time, first-pass biotransformation in the gastrointestinal tract (GIT) has been reported to limit the bioavailability of hydrophobic contaminants (such as polyaromatic hydrocarbons) in fish resulting in low BCFs compared to water-based exposure studies.[34]

4.2. Estimating k_M

Mackay[36] suggested a directly proportional relationship between the log BCF and log K_{OW} of hydrophobic substances with a log $K_{OW} < 10^6$ and BCFs > 10 and thermodynamic equilibrium between the concentration in the lipid phase (fish) and the water. This equilibrium partitioning model relies on the assumption that the elimination of hydrophobic substances is dominated by diffusion to the gills, *i.e.* that $k_T = k_2$. Since the model does not account for elimination through metabolic biotransformation, the model can be expected to overestimate the BCF of substances that are metabolised.[32]

In the experiment conducted for this study, an important criterion for the selection of benchmark substances was that they would not be metabolised during the duration of the experiment. *i.e.* They should be within the applicability domain of the Mackay model.[36] The results confirmed this assumption with a directly proportional relationship between the estimated BCF_{BM} and K_{OW} of the benchmark substances (as illustrated by a linear relationship with a slope of 1 in the plot of log BCF_{BM} *versus* log K_{OW} Fig. 2).

When comparing the log BCF of the cedarwood oil constituents and benchmark substances with their respective log K_{OW},

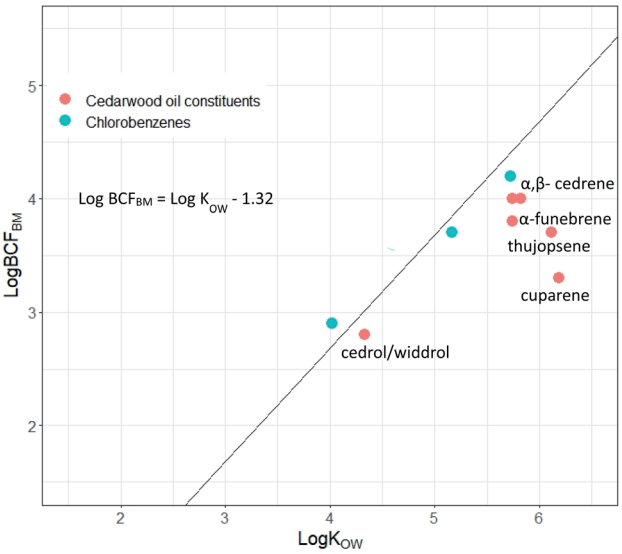

Fig. 2 Log BCF_{BM} *vs.* log K_{OW} of cedarwood oil constituents and benchmark substances. The black line represents the relationship between log K_{OW} and log BCF described by Mackay.[36]

all cedarwood oil constituents showed lower log BCFs than the benchmark substances of comparable K_{OW} (Fig. 2). As noted above, the low log BCFs of the cedarwood oil constituents compared to the log BCFs of the chosen benchmark substances indicate that the cedarwood oil constituents were eliminated through other mechanisms than just equilibrium partitioning (k_2). The data had been corrected for growth-dilution by the benchmarking, and therefore the additional elimination pathways could either be biotransformation (k_M) and/or excretion (k_E).

Arnot *et al.*[37] described the elimination rate constant for biotransformation (k_M) as:

$$k_M = k_T - (k_2 + k_E + k_G) = k_{TG} - (k_2 + k_E) \quad (2)$$

where k_E is the elimination rate constant for elimination through excretion [d^{-1}].

Chen *et al.*[22] found that k_E is negligible compared to k_2 and k_M. for HCB, PeCB as well as several constituents of pine oil. Based on the structural similarities between pine oil and cedarwood oil constituents (predominantly terpenoids),[38] we assume that this is true for cedarwood oil constituents as well. In this case eqn (2) can be simplified to

$$k_M = k_{TG} - k_2 \quad (3)$$

with this equation k_M for the cedarwood oil constituents was estimated using the measured k_{TG} and k_2 estimated from a Mackay equilibrium partitioning model[36] derived from the k_{TG} values of the benchmark chemicals (see ESI†). The resulting k_M for the measured cedarwood oil constituents in the experimental fish as well as in 10 g fish at 15 °C water temperature ($k_{M,N}$, normalized in accordance with the Arnot *et al.*[39] k_M database) are presented in Table 3.

Table 3 Derived biotransformation rate constant k_M [d^{-1}], total, growth-corrected elimination rate constant k_{TG} [d^{-1}], contribution of k_M to k_{TG} [%], and normalised biotransformation rate assuming 10 g fish weight and 15 °C water temperature $k_{M,N}$ [d^{-1}]

Chemical	k_M	k_{TG}	% k_M of k_{TG}	$k_{M,N}$
α-Cedrene	0.0022	0.013	17	0.004
β-Cedrene	0.0054	0.015	36	0.01
Thujopsene	0.023	0.029	79	0.041
Cuparene	0.061	0.067	92	0.11
Cedrol/widdrol	0.15	0.23	63	0.26
α-Funebrene	0.0092	0.02	46	0.017

The estimated contribution of biotransformation (k_M) to the total elimination rate (k_{TG}) differed substantially between different cedarwood oil constituents. k_M was practically equal to k_{TG} for thujopsene and cuparene, indicating that biotransformation is likely the dominant elimination pathway for these constituents (Table 3). The mixed aromatic – cyclo-aliphatic structure of cuparene (Table S1†) might make the cyclo-aliphatic part of cuparene susceptible to some initial biotransformation,[38] which the aliphatic sesquiterpenes (α-cedrene, β-cedrene) are not susceptible to. The cedrenes had a low contribution from biotransformation (<40%, Table 3), indicating that they were primarily eliminated *via* the gills. Cedrol/widdrol and α-funebrene seemed to be eliminated equally *via* gills and biotransformation (Table 3). There is very limited published literature on the potential biotransformation of cedarwood oil constituents. To the best of our knowledge, the only study published on the *in vivo* biotransformation of α-cedrene was performed on rats,[11] which limits the comparability of the results. Nevertheless, Kim *et al.*[11] reported indications for biotransformation of α-cedrene. They observed that α-cedrene was eliminated rapidly if injected into the blood stream of rats and were able to detect a hydroxylated metabolite, while no significant elimination was observed when the substance was administered through food.[11] This could be an indication that the low k_M for the cedrenes observed in our study might not be due to the inability of the fish's liver to biotransform these components but because they are not freely dissolved within the blood and therefore are not available for biotransformation in the liver within the time-frame of the experiment.[40] There is still very limited information regarding the bioaccumulation potential, biotransformation pathways (as well as persistence and toxicity) of essential oils in general. *In vitro* biotransformation-based studies such as the S9 liver assay (OECD test guideline 319B) or hepatocyte stability assay (OECD test guideline 319A) could potentially help to evaluate to what extend different cedarwood oil constituents can be biotransformed.

Our results indicate that major components of this essential oil might be bioaccumulative or very bioaccumulative according to the threshold of a BCF > 2000 and BCF > 5000, respectively. Further investigation of the bioaccumulative properties of essential oils is therefore warranted, as is discussion of how findings of bioaccumulative substances in natural products used for commercial purposes should be used in chemical assessment. Benchmarking can play a useful role in these investigations. Without benchmarking it was not possible to derive sufficiently constrained estimates of the BCFs for several of the major constituents in cedarwood oil, whereas with benchmarking it was shown that their BCFs lay close to the regulatory thresholds. Moreover, the utility of internal benchmarking for providing well-constrained BCF estimates in abbreviated dietary exposure studies on a chemical mixture, allows for the use of less test animals compared to a standard OECD305 test while maintaining the statistical robustness. Therefore, internal benchmarking can improve BCF studies with regards to mixture assessment, reduced test animal usage, and improved cost-effectiveness.

Conflicts of interest

There are no conflicts to declare.

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