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Biodegradation of an essential oil UVCB - Whole substance testing and constituent specific analytics yield biodegradation kinetics of mixture constituents

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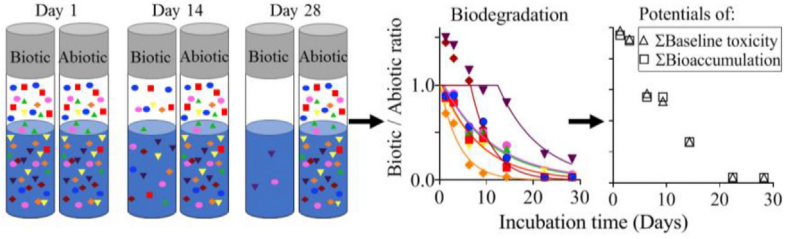
HIGHLIGHTS

- Whole UVCB testing yielded constituent specific primary biodegradation kinetics.
- Screening biodegradation of minor constituents using a non-targeted method.
- Estimated UVCB bioaccumulation and toxicity decreased during biodegradation.

GRAPHICAL ABSTRACT



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ABSTRACT

Testing and assessing the persistency, bioaccumulative and toxic properties of UVCBs (substances of Unknown or Variable composition, Complex reaction products or Biological materials) pose major technical and analytical challenges. The main aim of this study was to combine whole substance biodegradation testing with constituent specific analytics for determining primary biodegradation kinetics of the main UVCB constituents. An additional aim was to link the primary biodegradation kinetics of the main constituents to the bioaccumulation potential and baseline toxicity potential of the UVCB. Two closed biodegradation experiments were conducted using similar test systems but different analyses. The model substance, cedarwood Virginia oil, was tested at a low concentration and wastewater treatment plant effluent served as inoculum. We used microvolume solvent spiking for a quantitative mass transfer of the UVCB, while avoiding that co-solvent degradation would lead to anaerobic conditions. The biodegradation of UVCB constituents was determined with automated solid-phase microextraction coupled to GC-MS/MS using targeted analysis for main constituents and non-targeted analysis for minor constituents and non-polar degradation products. Primary biodegradation kinetics of main constituents, accounting for 73% w/w of the mixture, were successfully determined with degradation rate constants ranging from 0.09 to 0.25 d^{-1}. Minor constituents were also degraded and non-polar degradation products were not observed. Finally, the bioaccumulation potential and baseline toxicity potential of the mixture at test start were calculated and both parameters decreased then substantially.

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The strength of the new approach is the possibility of biodegradation testing of a whole UVCB at low concentration while generating constituent specific biodegradation kinetics.

1. Introduction

Importers and producers of chemicals are obligated to conduct risk assessment of the chemicals they market e.g. in the European Union and in the United States, in order to protect humans and the environment from hazardous chemical exposure (US Public law, 2002; EU Parliament and Council, 2006). It is of particularly high priority to test if chemicals are persistent, bioaccumulative and toxic (PBT) or very persistent and very bioaccumulative (vPvB). These properties of a substance are evaluated from standardized test methods or predicted from models (EU Parliament and Council, 2006; US EPA, 2020a). The standardized tests are not applicable for substances categorized as Unknown or Variable composition, Complex reaction products or Biological materials (UVCBs). Examples of UVCB substances are petroleum substances, biofuels and essential oils (Clark et al., 2013). Developing, applying and standardizing experimental procedures for UVCB substances is a challenge for the chemical industry and regulators, because UVCBs can consist of many thousands of constituents, the composition can vary between batches, and the constituents can have very different physicochemical and fate properties (Salvito et al., 2020; Clark et al., 2013; Sauer et al., 2020). Of the almost 12,000 substances registered under the EU regulation Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) in early 2017, around 20% are categorized as UVCB substances (ECHA, 2017c), and the same applies to around 25% of the approximately 68,000 substances on the non-confidential chemical substance list from the Unites States Environmental Protection Agency (US EPA) Toxic Substances Control Act Inventor (US EPA, 2020b). There is thus an urgent need for improved testing methods that can handle the major technical and analytical challenges that these substances pose.

The current PBT and vPvB assessment strategy for UVCBs in the EU is, that if the constituents of a UVCB are very similar with regard to their PBT-properties, the assessment can be based on a whole substance approach. In the whole substance approach, the UVCB is considered as one chemical substance for the purpose of the assessment and testing. If the constituents of the UVCB are not similar, the procedure is to test constituents or constituent fractions separately (ECHA, 2017b). When it is known which constituents are considered worst case in a PBT context, these constituents are individually assessed and used for the assessment of the whole UVCB. If the UVCB is so complex that it is not feasible to assess or identify all constituents, a fraction profiling approach can be applied. Here the UVCB is separated into fractions of constituents expected to have similar PBT properties. An assessment is then conducted either for the fraction or for a constituent considered characteristic of the specific fraction group (ECHA, 2017b).

Biodegradation of UVCB substances is the focus of this study, since biodegradation is the key removal process for many chemicals in the environment (Kowalczyk et al., 2015) and biodegradation data are of high importance in regulatory persistency assessment (ECHA, 2017a). A major disadvantage of the whole substance biodegradation testing approach is that test results may not be representative of all constituents. If whole substance test results indicate ready biodegradability, there will be a risk of missing potentially persistent constituents (ECHA, 2017b). The challenges related to the fraction profiling approach are that it requires a greater testing effort and that the individual substances or fractions may not be readily available for testing (ECHA, 2017b). Furthermore, as UVCB substances will be released to the environment as mixtures, some constituents may lead to enhanced or inhibited degradation of others e.g. by co-metabolism or competitive inhibition (Dalton and Stirling, 1982; NCGC, 2010). A consequence of testing constituents separately is that these mixture effects are not accounted for.

The present study combines whole UVCB biodegradation testing with a constituent specific analytical method. This addresses the disadvantages of the current approaches, while at the same time facilitates the generation of more data for UVCBs and their constituents. We applied an experimental platform that was developed for biodegradation testing of hydrophobic chemicals well below their solubilities in the ng/L to μg/L concentration range (Birch et al., 2017a, 2018). Test concentrations can be critical for the test results, and sufficiently low test concentrations are necessary in order to avoid dispersion of microdroplets, enzyme saturation and substrate toxicity (Hammershøj et al., 2019, Li and Mclachlan, 2019). This platform was very recently applied to test the mixture and concentration effect in biodegradation testing of composed and complex mixtures (Hammershøj et al., 2019, 2020b). The approach was further developed in the present study and then applied to obtain primary biodegradation kinetics data of the main constituents of a UVCB using a targeted analytical method. We used microvolume solvent spiking of only 1 μL for the quantitative transfer of a known UVCB mass to each test system while conserving the UVCB composition and avoiding that co-solvent degradation would lead to oxygen depletion and thus anaerobic conditions. As model UVCB we chose cedarwood Virginia oil that is mainly used in perfumery, toiletries and household chemicals (Baser and Buchbauer, 2010).

Fate-directed testing and risk assessment approaches for UVCBs are designed to account for fate processes such as biodegradation of constituents before evaluating their toxicity and bioaccumulative properties (Salvito et al., 2020). These approaches are especially relevant for substances that pass through Wastewater Treatment Plants (WWTPs), where the substances might be fully or partly biodegraded before entering the environment. The rationale behind this is that if chemicals rapidly degrade in WWTPs or in the receiving waters, then toxicity and bioaccumulation is of less concern. This approach is therefore relevant for fragrance materials, since 60% of these products are down-the-drain products (Bickers et al., 2003). The continuous use of products containing fragrance can lead to continuous environmental presence and potentially increased environmental exposure if these compounds are not degraded at the WWTP (Ceriani et al., 2015). The present study provides a new fate-directed method for screening of bioaccumulative and baseline toxic properties of UVCBs during the course of the biodegradation process based on primary biodegradation of main constituents. The constituent specific primary biodegradation kinetics are combined with partition coefficients to estimate the time resolved bioaccumulation potential and baseline toxicity potential of constituents and for the whole UVCB in the test. Baseline toxicity is a non-specific toxicity that is exerted when the partitioning of substances into the membrane of an organism reaches a critical concentration. Baseline toxicity has been observed

to start at concentrations in the range 40–160 $mmol_{substance}/kg_{membrane}$ and is only relevant when the chemical or UVCB does not exert specific toxicity (Wezel and Opperhuizen, 1995). Cedarwood Virginia oil, the UVCB used in this study, was found not to cause excess toxicity on *Daphnia magna* (Trac et al., 2021).

The main aim of the present study was to conduct whole UVCB biodegradation testing in the μg/L range and then to use constituent specific analytics to obtain primary biodegradation kinetics of UVCB mixture constituents. For this purpose, cedarwood Virginia oil was used as test substance and Wastewater Treatment Plant (WWTP) effluent was used as inoculum in a biodegradation experiment. Primary biodegradation kinetics of the main cedarwood Virginia oil constituents were quantified using targeted analysis. The biodegradation of minor constituents and the possible formation of non-polar degradation products were monitored using non-targeted analysis. The additional aim of this study was to link the obtained primary biodegradation kinetics to changes in bioaccumulation potential and baseline toxicity potential of the UVCB. This was done using lipid-water and membrane-water partitioning calculations.

2. Materials and methods

2.1. Materials

The test UVCB was cedarwood Virginia oil (CAS 8000-27-9/85085-41-2, Givaudan, UK), mainly consisting of sesquiterpene hydrocarbons and sesquiterpene alcohols. Methanol (HPLC-grade, VWR International, Denmark) was used as solvent. Ultrapure water was generated with an Elga Purelab flex water system (Holm & Halby, Denmark).

2.2. WWTP effluent inoculum

The inoculum was chosen based on a scenario of the wastewater treatment plant (WWTP) being the point of entry for essential oils to the environment but without simulating the treatment process as such. Therefore WWTP effluent was used as inoculum. It was sampled on the 5th of November 2019 from the Lynetten WWTP (Copenhagen, Denmark) which treats 57 million m^3/year. Lynetten mainly treats domestic wastewater and the effluent is not disinfected prior to discharge to the sea (Biofos WWTP, 2013, 2018). The sample was transported in an insulated bag and then stored at 15 °C. The experiment was started within 8 h after sampling, without any further treatment of the effluent sample.

Effluent temperature was 16.2 °C and the pH was 7.4, measured at the WWTP. A heterotrophic plate count of the sample yielded $1.0 \cdot 10^3$ and $6.9 \cdot 10^3$ colony forming units/mL after 24 h and 72 h incubation at 20 °C respectively on R2A agar (DS/EN ISO 8199). This heterotrophic plate count is comparable to those found in lake and stream-water in an earlier study (Birch et al., 2017b). The three most abundant genera in the WWTP effluent, based on DNA sequencing, were the *12up* belonging to the Betaproteobacteria class, and *Candidatus Planktoluna* and *Candidatus Rhodoluna* belonging to the Actinobacteria class (DNA sequencing methodology and results are provided in SI 1, Tables S1–S3). Further sample characterization included total suspended solids, non-volatile organic carbon and conductivity (see SI 2, Table S4).

2.3. Biodegradation testing

Two parallel biodegradation tests based on substrate depletion were performed using similar test systems but different analyses. The purpose of the first biodegradation test was to obtain primary biodegradation kinetics of eight main cedarwood oil constituents that together make up 73% by weight of the mixture (see Table 1). Primary biodegradation was evaluated based on targeted analysis of triplicate biotic and abiotic test systems on day 1, 3, 6, 9, 14, 22 and 28 of incubation (42 test systems in total). The purpose of the second biodegradation test was to investigate overall trends for the minor mixture constituents and observe potential formation of non-polar degradation products. This was based on non-targeted analysis of triplicate biotic and abiotic test systems on day 1, 6, 15 and 29 of incubation (24 test systems in total). The formation of polar metabolites was not included in the present study, since it requires other analytical approaches.

The test systems were prepared in 20 mL gas tight vials in amber glass (Mikrolab Aarhus, Denmark) with magnetic screw caps and a silicone/PTFE septum (Sigma-Aldrich, Denmark). Biotic test systems were prepared by adding 10 mL WWTP effluent to the vials and spiking with 1 μL stock solution of cedarwood oil in methanol to a final cedarwood oil concentration of 57 μg/L. Abiotic test systems were prepared in the same way, but using ultrapure water instead of the WWTP effluent. The tested concentration of cedarwood oil corresponds to 0.01–15.2% of the estimated solubility of the main constituents, thus all main constituents were dissolved in the test system (see SI 3, Table S5). The test systems were spiked using a gas tight 'eVol XR' automatic syringe (SGE Analytical Science, Australia) and were closed immediately after spiking. Solvent spiking was chosen as dosing method in order to transfer a defined mass to the test systems while conserving the composition of the cedarwood oil. Initial tests with 0.03% methanol (vol:vol) and 25% headspace resulted in oxygen depletion and anaerobic conditions during the course of the experiment (results not presented). The oxygen demand of the methanol was therefore estimated before the next experiment and the headspace was dimensioned to provide a sufficient oxygen reservoir (see SI 6). With a solvent addition minimized to 0.01% methanol (vol:vol), a 50% headspace was deemed sufficient. The aerobic conditions were confirmed by oxygen measurements throughout the experiment. The test systems were incubated at 12 ± 0.5 °C on tube roller mixers at ~30 rounds per minute for the duration of the biodegradation experiments (up to 29 days).

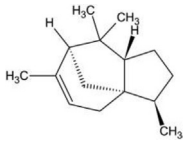
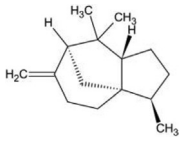
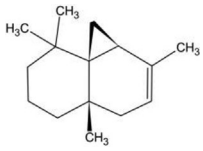
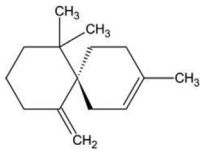
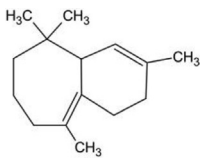
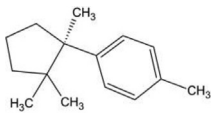
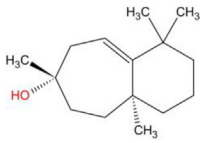
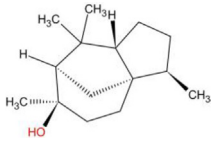
2.4. Chemical analysis

After incubation the test systems were moved to a PAL RTC 120 autosampler (CTC, Zwingen, Switzerland) for analysis without any manual sample preparation steps. Automated solid phase microextraction (SPME) was performed using a 7 μm bonded polydimethylsiloxane (PDMS) fiber (Supelco, USA). Separation and detection of the cedarwood oil constituents were done by Gas Chromatography (GC) coupled to triple quadrupole Mass Spectrometry (MS/MS) (7890B GC system/7010B GC/TQ, Agilent Technologies, Denmark). The GC column (Agilent Technologies 122-5562 UI) was 60 m long with a 250 μm inner diameter and 0.25 μm film thickness and helium was used as carrier gas at 1.2 mL/min. In the first biodegradation experiment, the test systems were analysed with a Multiple Reaction Monitoring (MRM) method. In the second biodegradation experiment, the test systems were analysed with a scan (MS2 scan) method. Details on the SPME-GC-MS/MS methods are provided in SI 4.

2.5. Identification of main constituents in cedarwood Virginia oil

Prior to the biodegradation tests, the main constituents in the cedarwood Virginia oil were analysed using SPME-GC and MS/MS in scan mode (method described in SI 4). Compounds were identified from the obtained ion spectra using spectral library match search in NIST (17) in the software "Agilent MassHunter Unknown

Table 1
The fraction, molecular structure and physical- and chemical properties of the main constituents of the tested cedarwood Virginia oil. K_{ow} is the octanol-water partitioning coefficient.

Compound name and CAS No.[a,b]	Fraction in Cedarwood Virginia oil (%)[b]	Molecular structure	Chemical formula[a]	Molecular weight (g/mol)[a]	Water solubility from K_{ow} (mg/L)[c]	Log (K_{ow})[c]
α-Cedrene 469-61-4	23.0		$C_{15}H_{24}$	204.36	0.15	5.7
β-Cedrene 546-28-1	4.57		$C_{15}H_{24}$	204.36	0.13	5.8
Thujopsene 470-40-6	19.1		$C_{15}H_{24}$	204.36	0.072	6.1
β-Chamigrene 18431-82-8	0.96		$C_{15}H_{24}$	204.35	0.012	7.0
β-Himachalene 1461-03-6	1.68		$C_{15}H_{24}$	204.35	0.045	6.4
Cuparene 16982-00-6	1.45		$C_{15}H_{22}$	202.33	0.22	6.2
Widdrol 6892-80-4	1.80		$C_{15}H_{26}O_1$	222.37	7.9	4.8
Cedrol 77-53-2	20.6		$C_{15}H_{26}O_1$	222.37	22.0	4.3

[a] U.S. National Library of Medicine (2019).
[b] From composition analysis made by Givaudan.
[c] National Food Institute DTU, 2019.

Analysis" after deconvolution. The fraction of each of the main constituents in the cedarwood oil was determined by liquid injection of a dilution of the cedarwood oil in hexane using GC coupled to a flame ionization detector (FID) (method description in SI 5). The results of identification and fraction of main constituents were compared to the composition analysis on the same batch of cedarwood oil provided by Givaudan, where a similar analytical approach had been used.

In the present study it was possible to detect and identify eight main constituents in the cedarwood Virginia oil: α-cedrene, cedrol, thujopsene, β-cedrene, β-chamigrene, β-himachalene, cuparene and widdrol (ion spectra compared to spectra library match provided in SI 5, Fig. S1). These identifications were confirmed by the analysis from Givaudan. The fraction analyses were also comparable (see SI 5, Table S8), but the analysis from Givaudan was based on a longer GC program and therefore the constituents were separated better and the fraction analysis was more accurate. Throughout this study, the fraction estimations of the main constituents provided by Givaudan were used (presented in Table 1). The present study covers all constituents in the tested batch of cedarwood Virginia oil

with a fraction >1%. The exception was β-funebrene with a fraction of 2% (see SI 5, Table S8), which could not be separated from α-cedrene in the MS/MS analysis. The molecular structure of the eight main constituents and their physical- and chemical properties are presented in Table 1.

2.6. Data treatment: main constituents

Quantitative analysis was performed using the software 'Agilent MassHunter Quantitative Analysis (for QQQ)', to obtain peak areas for all eight main constituents from triplicate biotic and abiotic test systems per measuring day. Biotic removal was calculated for each constituent as the relative peak area, $PA_{relative}$ (Eq. (1)) by dividing the peak area in a biotic test system, PA_{biotic}, with the peak area from an abiotic test system, $PA_{abiotic}$, analysed immediately after the biotic test system.

$$PA_{relative} = \frac{PA_{biotic}}{PA_{abiotic}} \quad (1)$$

A first order degradation model with lag-phase (Eq. (2)) was fitted to the data in order to estimate the first order degradation rate constant in the test system, k_{system}, and the lag phase, t_{lag}, for each of the main constituents. The model fit was done in GraphPad Prism v.8.

$$PA_{relative}(t) = \begin{cases} 1 & for\ t < t_{lag} \\ e^{-k_{system}(t-t_{lag})} & for\ t \geq t_{lag} \end{cases} \quad (2)$$

For each of the main constituents the half-life, $T_{½,system}$, was calculated from Eq. (3).

$$T_{\frac{1}{2},\,system} = \frac{\ln(2)}{k_{system}} \quad (3)$$

2.7. Data treatment: minor constituents and degradation products

For each measuring day, triplicate Total Ion Chromatograms (TIC) from the scan method of biotic test systems were compared to triplicate TICs of abiotic test systems in the software 'Agilent MassHunter Qualitative Analysis Navigator' by overlay of the chromatograms. In the retention time (RT) interval where cedarwood oil constituents were present, 18.5–21.0 min, the heights of minor peaks were compared, in order to investigate if minor constituents were biodegraded during the course of the biodegradation test. Furthermore, it was investigated if new peaks or an increase of existing peaks could be observed in the entire chromatogram of the biotic test systems, which could be caused by formation of non-polar biodegradation products.

2.8. Quality Assurance

Background response. Background response in the WWTP effluent was measured by SPME-GC-MS/MS (MRM method) in 7 vials containing 10 mL non-spiked WWTP effluent immediately after sampling. A quantitative analysis was performed in the retention time interval for each of the main constituents. The response in the non-spiked WWTP effluent was below 1% of the response of the initial spiked concentration for all main constituents (see SI 6, Table S12).

Blanks. Blank test systems with 10 mL ultrapure water were incubated along with the abiotic and biotic test systems in order to check for potential contamination during incubation of the test systems. At each analysis run, one of the incubated blanks and blanks prepared the same day were included. The response in all blanks was two to three orders of magnitude below the response in the standards at the initial spiked concentration.

Temperature and oxygen. Test systems were incubated at 12 °C in a Binder KB 400 cooling incubator (Binder, Germany). The temperature in the incubator was confirmed throughout the experiment by an Easylog USB temperature datalogger, which showed only transient temperature variations of 0.5–1 °C due to opening the incubator when vials were collected for analysis (see SI 6, Fig. S2). The oxygen content in the test systems was measured at set time intervals with a PreSens oxygen meter (Microx 4, Germany) using two different methods: a needle sensor and contactless oxygen sensor spots. The initial oxygen concentration was 10.5 mg/L (20 °C). This concentration was stable in the abiotic test systems throughout the experiment, whereas in the biotic test systems it decreased during the first 14 days to a concentration around 7 mg/L at which the concentration became stable. Throughout the experiment there were thus aerobic conditions in both abiotic and biotic test systems (see SI 6, Fig. S2).

Calibration series. Calibration series corresponding to 3.3%, 10%, 30% and 100% of the initial spiked concentration in the test systems were analysed each measuring day, before and after the series of triplicate biotic and abiotic test systems. The calibrants were prepared in 20 mL vials with 10 mL ultrapure water spiked with 1 μL stock solution of cedarwood Virginia oil in methanol. On day 1, 3, 6, 9, 14, 22 and 28 the calibrants were analysed with the targeted analytical method (SPME-GC-MS/MS MRM method, described in SI 4). On day 15 and 29 the calibrants were analysed with the non-targeted analytical method (SPME-GC-MS/MS scan method, described in SI 4).

Non-linear calibration curves. The relationship between peak areas and concentrations was not fully linear for all constituents. The impact of this non-linearity on the determination of biodegradation kinetics was thus investigated for the three major constituents, α-cedrene, thujopsene and cedrol, using the calibration curves for converting from peak area to concentration, as described in SI 6. It was found that the correction of the non-linear response resulted in biodegradation kinetics very similar to those based on peak area ratios (see SI 6, Fig. S4 and Table S13), and the results presented in this study were thus based on peak area ratios.

Quantification limit. The lowest calibrant, 3.3%, was used as quantification limit, and thus relative peak areas below 0.033 were assigned the value 0.033. Due to decreasing instrument sensitivity for widdrol on day 22 and 28, no peak was observed for the 3.3% calibrant, the 10% calibrant and for two out of the three biotic test systems on both days. Since a peak was detected for the 30% calibrant, biotic test systems with no peak observed are known to have less than 30% of the initial spiked concentration left. When no peak was observed for widdrol in a biotic test system, the relative peak area was therefore assigned the value 0.1 on those two days.

Correction for abiotic losses. Abiotic losses of the main constituents were determined based on constituent specific peak areas in the abiotic test systems (n = 3) relative to freshly spiked vials (n = 2). The highest loss was observed for β-himachalene, where the concentration in the abiotic test system decreased 74% during the experiment. For α-cedrene, β-cedrene, thujopsene, β-chamigrene and cuparene losses were 25–55%. No abiotic losses were observed for the more polar constituents, widdrol and cedrol (results of abiotic losses are provided in SI 6, Fig. S5). The observed losses can be due to diffusion through the septum, sorption to test system or abiotic transformation processes (Nagahama and Tazaki, 1987). Such abiotic losses should be kept at a minimum and were in the present study corrected for by using the peak area ratio between biotic test system and abiotic controls (Eq. (1)).

2.9. Bioaccumulation potential and baseline toxicity potential calculations

It was investigated how the primary biodegradation of main constituents in the water phase of the test systems impacted the bioaccumulation potential and the baseline toxicity potential of the mixture. These calculations reflect the equilibrium partitioning of the main cedarwood oil constituents into the lipid of an aquatic organism (bioaccumulation potential) and into the membrane of an aquatic organism (baseline toxicity potential). The possible biotransformation in the organism was thus not accounted for. The initial concentration of a constituent, *i*, in the water phase, $C_{water,i,0}$, of the test system was calculated from Eq. (4).

$$C_{water,i,0} = \frac{m_{cw} \cdot f_i \cdot f_{water,i}}{V_{water}} \quad (4)$$

where m_{cw} is the mass of cedarwood oil added to the test system, f_i is the fraction of constituent *i* in the cedarwood oil (Table 1), V_{water} is the volume of water in the test systems and $f_{water,i}$ is the fraction of the constituent *i* in the water phase calculated from partitioning between headspace and water phase Eq. (5)

$$f_{water,i} = \frac{1}{1 + K_{air/water,i} \cdot \frac{V_{air}}{V_{water}}} \quad (5)$$

$K_{air/water,i}$ is the air-water partitioning coefficient of the constituent *i* (see Table 2), and V_{air} is the volume of air in the test system.

The initial concentration of constituent *i* in water was converted to the corresponding equilibrium concentration in storage lipid, for bioaccumulation potential estimations, and in membrane lipid, for baseline toxicity potential estimations by multiplying with the respective lipid-water, $K_{lipid/water}$, and membrane-water, $K_{membrane/water}$, partitioning coefficients. Partition coefficients from the UFZ-LSER database are presented in Table 2 (Ulrich et al., 2017; Goss, 2019). The change in concentration of a constituent in the lipid and membrane over time caused by biodegradation, $C(t)$, were found using the average of the triplicate relative peak areas, $\overline{PA_{relative,i}}$, at the measuring day, *t*, according to Eq. (6) and Eq. (7).

$$C_{lipid,i}(t) = C_{water,i,0} \cdot K_{lipid/water,i} \cdot \overline{PA_{relative,i}}(t) \quad (6)$$

$$C_{membrane,i}(t) = C_{water,i,0} \cdot K_{membrane/water,\ i} \cdot \overline{PA_{relative,i}}(t) \quad (7)$$

The bioaccumulation potential and baseline toxicity potential of the cedarwood oil mixture (disregarding minor constituents and degradation products) were then estimated as the sum concentration of the main constituents in storage lipid and membrane lipid respectively at each measuring day. The baseline toxicity was calculated in the unit mmol/kg assuming a membrane density of 0.9 kg/L, and the bioaccumulation potential was calculated in the unit g/kg assuming a lipid density of 0.9 kg/L. The results of the calculated concentration of the cedarwood oil mixture (disregarding minor constituents and degradation products) in lipid and membrane were plotted using GraphPad Prism v.8.

Table 2
Air-water, $K_{air/water}$, membrane-water, $K_{membrane/water}$, and lipid-water, $K_{lipid/water}$ partitioning coefficients modelled by the UFZ LSER Database for the eight main constituents in the tested cedarwood Virginia oil. RMSE for $K_{lipid/water}$ is 0.4–0.5 log units and for $K_{membrane/water}$ 0.8–1 log unit (Ulrich et al., 2017; Goss, 2019).

	$K_{air/water}$ (L_{water}/L_{air})	$K_{membrane/water}$ ($L_{water}/L_{membrane}$)	$K_{lipid/water}$ (L_{water}/L_{lipid})
α-Cedrene	5.1	$1.2 \cdot 10^6$	$9.5 \cdot 10^6$
β-Cedrene	22	$3.6 \cdot 10^6$	$3.2 \cdot 10^7$
Thujopsene	0.45	$6.2 \cdot 10^5$	$2.5 \cdot 10^6$
β-Chamigrene	0.46	$7.9 \cdot 10^5$	$3.6 \cdot 10^6$
β-Himachalene	0.66	$6.0 \cdot 10^5$	$2.6 \cdot 10^6$
Cuparene	0.0040	$3.7 \cdot 10^4$	$8.7 \cdot 10^4$
Widdrol	$7.6 \cdot 10^{-5}$	$6.6 \cdot 10^3$	$4.4 \cdot 10^3$
Cedrol	0.0049	$9.8 \cdot 10^4$	$1.4 \cdot 10^5$

3. Results and discussion

3.1. Primary biodegradation kinetics of main constituents

A first order degradation model with lag phase fitted to experimental biodegradation data for each of the eight main constituents in cedarwood Virginia oil are shown in Fig. 1. More than 90% primary biodegradation was observed within the 28 day test duration

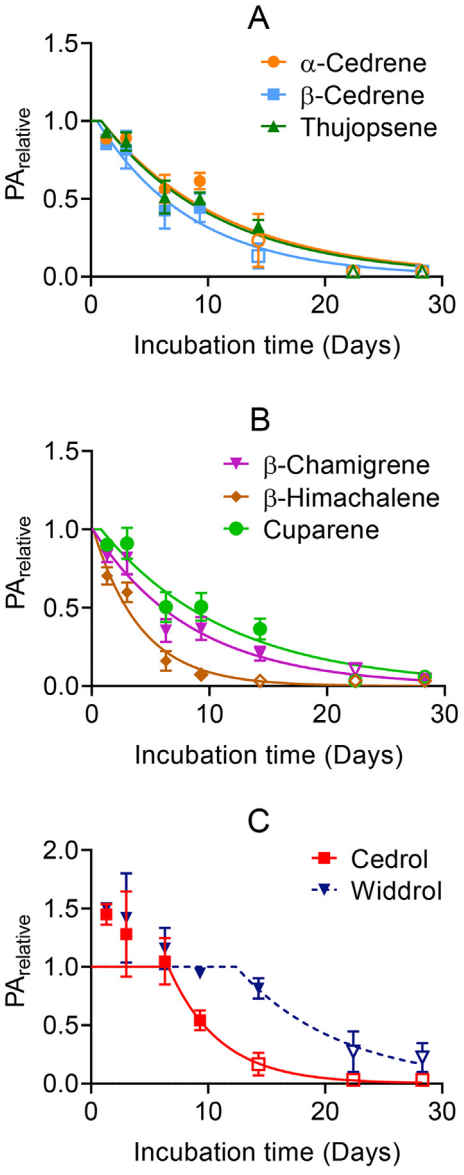


Fig. 1. Biodegradation results for the eight main constituents in the tested cedarwood oil. Mean relative peak areas, $PA_{relative}$, with standard error of mean (SEM), n = 3. Open symbols indicate one or more $PA_{relative}$ below limit of quantification. A first order degradation model with lag-phase was fitted to the data. Due to decrease in instrument sensitivity on day 22 and 28 the model fit for widdrol is uncertain, indicated by a dotted line.

for the constituents: α-cedrene, β-cedrene, thujopsene, β-chamigrene, β-himachalene, cuparene and cedrol. More than 50% primary biodegradation was seen in all replicates on day 28 for widdrol. Widdrol may have been degraded even more, but a more exact extent of degradation could not be quantified due to a decrease in instrument sensitivity on the MS/MS on day 22 and 28. Widdrol had the lowest response of the main constituents because widdrol only constitutes ~2% of cedarwood oil and is more polar leading to lower enrichment during SPME extraction. The first-order degradation rate constants were found to be within the same order of magnitude for all eight main constituents, 0.09–0.25 d^{-1}, resulting in test system half-lives of 2.8–7.5 days (see SI 7, Table S14). Constituents with air-water partitioning coefficients >1 L/L have much higher first order biodegradation rate constants in the water phase than determined for the test systems because the headspace acts as a buffer replenishing the water phase when degradation occurs (Birch et al., 2017a). Test system biodegradation rate constants were converted to water phase biodegradation rate constants using air-water partitioning coefficients according to Birch et al. (2017a) (see SI 8). This conversion led to markedly higher rate constants for α-cedrene and β-cedrene, which however is associated with some uncertainty related to the estimation of partition coefficients. To our knowledge, biodegradation kinetics data have not previously been published for cedarwood oil or its constituents, but previous studies have investigated their ready biodegradability (ECHA, 2011; 2018; Jenner et al., 2011). The results of the previous published studies are in agreement with the findings in the present study, that main cedarwood oil constituents are biodegradable (see SI 9).

In the present study α-cedrene, β-cedrene, thujopsene, β-chamigrene, β-himachalene and cuparene had a short lag-phase < 1 day (see Fig. 1). The short lag-phase indicates that bacteria able to degrade these constituents were present in sufficient numbers in the effluent from the WWTP. Bacterial growth and adaptation were therefore not necessary for initiating the biodegradation of these cedarwood oil constituents. Widdrol had a lag-phase of 12.4 days and cedrol of 6.6 days. The model fit of the lag-phase was poor for these two constituents, since the response in the biotic test systems was 1.5 times higher compared to the response in the abiotic test systems after one day of incubation, where it was expected that the response from biotic and abiotic test systems would be equal. We have found no explanation for this observation.

3.2. Biodegradation of minor constituents and degradation products

Biodegradation of minor constituents was evident in the second experiment, since chromatogram peaks of minor constituents decreased much faster in biotic than abiotic test systems (Fig. 2). This was confirmed by comparing chromatograms at the end of the experiment, where peaks were still observed in the abiotic test systems, whereas all peaks in the biotic test systems were on the same level as the blanks (see SI 10, Figs. S6 and S7). No biodegradation products were observed during the 29 days of the experiment since no increase of existing peaks or occurrence of new peaks were observed in the chromatograms from the biotic test systems (see SI 10, Fig. S8). Any possible degradation products were thus too polar or produced at too low concentrations to be detected by SPME-GC-MS/MS.

3.3. Bioaccumulation potential and baseline toxicity potential

The calculations of the change in bioaccumulation potential and baseline toxicity potential of the cedarwood oil mixture were based on the primary biodegradation of the eight main constituents

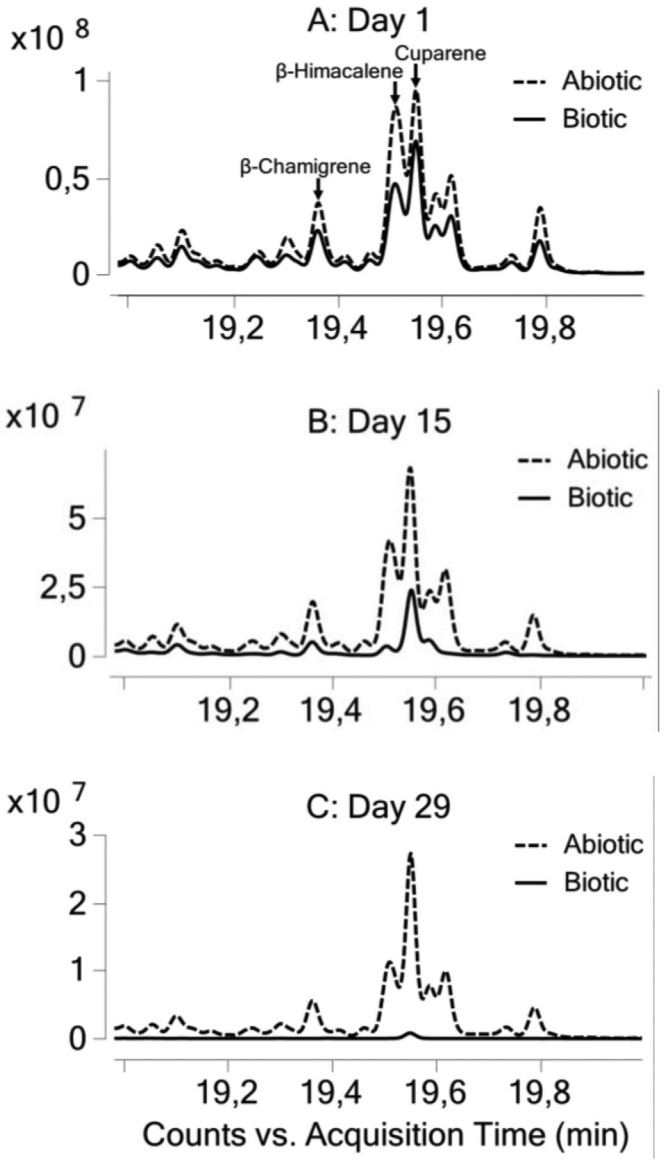


Fig. 2. Biodegradation scan results for minor constituents in the tested cedarwood oil. A sub-section of the full chromatogram chosen to illustrate the disappearance of the minor peaks. Three of the peaks are included in the MRM method (β-chamigrene, β-himachalene and cuparene), the remaining peaks are not. Results from one biotic test system (solid line) compared to one abiotic test system (dotted line) on day 1 (A), day 15 (B) and day 29 (C).

during incubation. Minor constituents and degradation products were not accounted for. We expect that their contribution to the overall bioaccumulation potential and baseline toxicity potential of the mixture is low, since minor constituents were found to be degraded, non-polar degradation products were not observed, and polar chemicals have lower $K_{lipid/water}$ and $K_{membrane/water}$. Seven of the eight main constituents were more than 90% degraded during the biodegradation experiment, which resulted in a substantial decrease of the bioaccumulation potential and baseline toxicity potential (Fig. 3). The largest decrease was observed from day 3 to day 22 of incubation. The uncertainty related to the model-predicted lipid-water partitioning coefficients (0.4–0.5 log units) and membrane-water partitioning coefficients (0.8–1 log units) (Goss, 2019) for each cedarwood oil constituent is higher than the uncertainties related to the biodegradation measurement. This

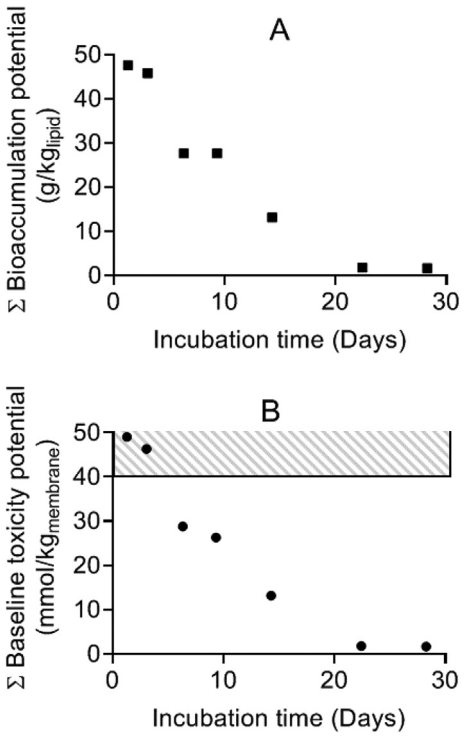


Fig. 3. Decline in bioaccumulation potential (A) and baseline toxicity potential (B) of cedarwood oil, during primary biodegradation of main constituents. The baseline toxicity interval (40-160 mmol/kg$_{membrane}$) is marked as the grey striped area in Figure B (Van Wezel and Opperhuizen, 1995).

implies a higher precision and accuracy for the temporal changes of baseline toxicity and bioaccumulation potential than for their absolute values.

The initial contribution of each constituent to baseline toxicity and bioaccumulation potential was evaluated from their air/water, membrane/water and lipid/water partition coefficients (Table 2) and from the initial fraction of each constituent (Table 1). The constituents with the calculated highest contribution to the bioaccumulation potential of the cedarwood oil mixture were α-cedrene and thujopsene, both contributing ~40% at the start of the experiment whereas β-cedrene and cedrol contributed 6-7% and the remaining constituents contributed <3% each. Thujopsene had the highest contribution to the baseline toxicity potential, contributing ~50% at the start of the experiment, while α-cedrene contributed 26% and cedrol 17%. Since α-cedrene and thujopsene, which had the highest contribution to both potentials, degraded at comparable rates within 28 days and had short lag-phases, the decrease in bioaccumulation potential and baseline toxicity potential shown in Fig. 3 was similar to the biodegradation curves of these constituents in Fig. 1.

The initial baseline toxicity potential calculated from the spiked concentration in the present study is potentially within the lower range of the baseline toxicity interval of 40−160 mmol/kg$_{membrane}$ (Wezel and Opperhuizen, 1995) (see Fig. 3B). This level was not seen to prevent the microorganisms in the test systems from degrading the cedarwood oil (Fig. 1). Particularly the general absence of significant lag-phases suggests that the test concentration was below an inhibitory level (Hammershøj et al., 2019). Reduced sensitivity to baseline toxicity in degrader microorganisms compared to larger organisms have been reported earlier (Winding et al., 2019).

3.4. Improvements of UVCB testing and assessment

The testing method suggested in the present study deals with several major challenges of whole substance testing and provides an improved approach for testing biodegradation of UVCBs. There are several advantages of whole UVCB substance testing combined with a targeted Multiple Reaction Monitoring (MRM) and a non-targeted scan method. Constituent specific primary biodegradation kinetics can be obtained with the MRM method where it is certain that the kinetics apply to the identified constituent. Testing on the whole substance combined with constituent specific analytics makes it possible to produce data more efficiently for UVCBs, avoiding the large number of tests required for single constituent and fraction testing, and does at the same time account for possible mixture effects. Minor constituent that are not degraded can be discovered from the non-targeted analysis, which is usually a challenge when testing on whole UVCB substances.

The present study used solvent spiking as dosing method, in order to quantitatively transfer the UVCB into solution. To some degree this resembles the scenario where essential oils contained in cleaning or wash-off products are disposed to the sewer systems and transported to the WWTP. By changing the dosing method, it can be adapted to simulate different modes of entry (Hammershøj et al., 2020a). In the test systems, the solvent concentration was kept at an absolute minimum and well below the levels that are used and accepted in aquatic toxicity testing. While the methanol will add some co-substrate, no toxicity or inhibition of the microbial population is expected at 0.01% methanol.

In the EU regulation it is required that the PBT assessment of UVCBs covers all constituents present in concentration of ≥0.1% (ECHA, 2017b). The present study only covered cedarwood oil constituents of 1% or higher with the targeted analysis. It is possible to develop the method to also include constituents with fractions below 1%, but this will vary from one UVCB to another and is also dependent on how appropriate the analytical method is for the UVCB. Further, for a given natural complex substance, the constituents are generally structurally related as a consequence of the plant's biochemistry. Therefore, any unidentified constituents present at < 1% will often have similar PBT properties to the known constituents (EFEO/IFRA, 2016). This is supported by the results presented in this paper, which showed that the unidentified minor components were also degraded. Biodegradation kinetics of constituents could also be obtained from non-targeted analysis methods, which has recently been done covering 104 diesel oil constituents during whole substance testing (Hammershøj et al., 2020b). Identifying all constituents of very complex UVCBs prior to testing can be very time consuming and tedious work. In this case it can be further investigated whether it is practical and sound assessing biodegradation of minor constituents by first conducting a whole substance biodegradation experiment and at the end identifying those constituents that did not degrade.

A limitation of the targeted analytical method is that only primary biodegradation is assessed. The present study attempted to address this by investigating occurrence of non-polar degradation products with the scan method, but none were observed. It is possible that the degradation products were too polar to be detected by SPME-GC-MS/MS or that degradation products are transient because the constituents are completely mineralized. Since degradation products can be persistent and toxic, these need to be considered as well, which for the case of cedarwood oil would require additional analysis, such as LC-MS, that covers more polar compounds. Polar substances are usually less bioaccumulative, contribute less to baseline toxicity but can still have a specific mode of toxic action. The method used in this study could be further developed, e.g. by applying different analytical platforms to an

assessment of what is remaining at the end of the biodegradation experiment. If any degradation products are found, a screening of their bioaccumulation and toxicity properties could be done in order to evaluate if further testing is needed.

4. Conclusion

This study introduced an improved biodegradation testing method for essential oil UVCBs by combining whole substance testing with a targeted analytical method. The method was tested on cedarwood Virginia oil, where primary biodegradation kinetics were successfully obtained for eight main constituents: α-cedrene (23%), cedrol (21%), thujopsene (19%) β-cedrene (5%), β-chamigrene (1%), β-himachalene (2%), cuparene (1%) and widdrol (2%). First-order degradation rate constants of these main constituents ranged between 0.09 and 0.25 d^{-1}. The non-targeted GC-MS/MS analysis showed biodegradation of many minor constituents. For some UVCBs it will be possible to apply a more comprehensive analytical approach, which then can yield primary biodegradation kinetics of more minor constituents. Due to the complexity of UVCBs, that can have many thousands of constituents with varying physicochemical and fate properties, testing on the whole substance will be advantageous for more efficiently obtaining data for UVCBs and constituents while accounting for mixture effects. Additionally, time resolved bioaccumulation and baseline toxicity potential of UVCBs can be estimated from primary biodegradation kinetics and partitioning coefficients, where both whole substance effects and the difference in fate properties between constituents are accounted for. For cedarwood oil, the primary biodegradation of main constituents resulted in a substantial decrease of the bioaccumulation potential and baseline toxicity potential. Further research should be directed to cover a broader range of UVCBs and UVCB constituents, and also biodegradation products, which likely will require new combinations of sampling and analytical methods.

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Credit author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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