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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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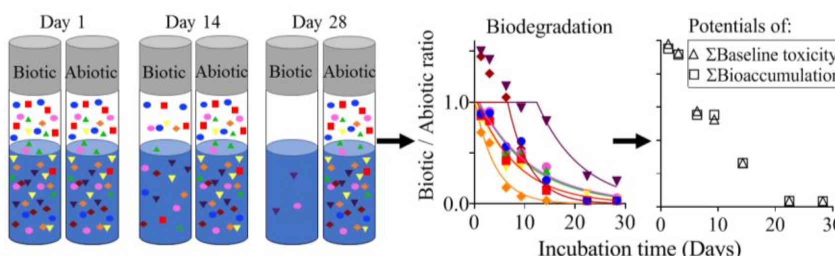
Graphical abstract



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 0.09-0.25 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. 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.

37 **1. Introduction**

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

58 The current PBT and vPvB assessment strategy for UVCBs in the EU is, that if the constituents of
59 a UVCB are very similar with regard to their PBT-properties, the assessment can be based on a

60 whole substance approach. In the whole substance approach, the UVCB is considered as one chem-
61 ical substance for the purpose of the assessment and testing. If the constituents of the UVCB are
62 not similar, the procedure is to test constituents or constituent fractions separately (ECHA, 2017b).
63 When it is known which constituents are considered worst case in a PBT context, these constituents
64 are individually assessed and used for the assessment of the whole UVCB. If the UVCB is so
65 complex that it is not feasible to assess or identify all constituents, a fraction profiling approach
66 can be applied. Here the UVCB is separated into fractions of constituents expected to have similar
67 PBT properties. An assessment is then conducted either for the fraction or for a constituent con-
68 sidered characteristic of the specific fraction group (ECHA, 2017b).

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

81 The present study combines whole UVCB biodegradation testing with a constituent specific ana-
82 lytical method. This addresses the disadvantages of the current approaches, while at the same time

83 facilitates the generation of more data for UVCBs and their constituents. We applied an experi-
84 mental platform that was developed for biodegradation testing of hydrophobic chemicals well be-
85 low their solubilities in the ng/L to µg/L concentration range (Birch *et al.*, 2017a, 2018). Test
86 concentrations can be critical for the test results, and sufficiently low test concentrations are nec-
87 essary in order to avoid dispersion of microdroplets, enzyme saturation and substrate toxicity
88 (Hammershøj *et al.*, 2019, Li and Mclachlan, 2019). This platform was very recently applied to
89 test the mixture and concentration effect in biodegradation testing of composed and complex mix-
90 tures (Hammershøj *et al.*, 2019, 2020b). The approach was further developed in the present study
91 and then applied to obtain primary biodegradation kinetics data of the main constituents of a
92 UVCB using a targeted analytical method. We used microvolume solvent spiking of only 1 µL for
93 the quantitative transfer of a known UVCB mass to each test system while conserving the UVCB
94 composition and avoiding that co-solvent degradation would lead to oxygen depletion and thus
95 anaerobic conditions. As model UVCB we chose cedarwood Virginia oil that is mainly used in
96 perfumery, toiletries and household chemicals (Baser and Buchbauer, 2010).

97 Fate-directed testing and risk assessment approaches for UVCBs are designed to account for fate
98 processes such as biodegradation of constituents before evaluating their toxicity and bioaccumu-
99 lative properties (Salvito *et al.*, 2020). These approaches are especially relevant for substances that
100 pass through Wastewater Treatment Plants (WWTPs), where the substances might be fully or
101 partly biodegraded before entering the environment. The rationale behind this is that if chemicals
102 rapidly degrade in WWTPs or in the receiving waters, then toxicity and bioaccumulation is of less
103 concern. This approach is therefore relevant for fragrance materials, since 60% of these products
104 are down-the-drain products (Bickers *et al.*, 2003). The continuous use of products containing
105 fragrance can lead to continuous environmental presence and potentially increased environmental

106 exposure if these compounds are not degraded at the WWTP (Ceriani *et al.*, 2015). The present
107 study provides a new fate-directed method for screening of bioaccumulative and baseline toxic
108 properties of UVCBs during the course of the biodegradation process based on primary biodegra-
109 dation of main constituents. The constituent specific primary biodegradation kinetics are combined
110 with partition coefficients to estimate the time resolved bioaccumulation potential and baseline
111 toxicity potential of constituents and for the whole UVCB in the test. Baseline toxicity is a non-
112 specific toxicity that is exerted when the partitioning of substances into the membrane of an or-
113 ganism reaches a critical concentration. Baseline toxicity has been observed to start at concentra-
114 tions in the range 40-160 $mmol_{substance}/kg_{membrane}$ and is only relevant when the chemical or UVCB
115 does not exert specific toxicity (Wezel and Opperhuizen, 1995). Cedarwood Virginia oil, the
116 UVCB used in this study, was found not to cause excess toxicity on *Daphnia magna* (Trac, 2019).

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

127 ## 2. Materials and methods

128 *2.1. Materials*

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

133 *2.2. WWTP effluent inoculum*

134 The inoculum was chosen based on a scenario of the wastewater treatment plant (WWTP) being
135 the point of entry for essential oils to the environment but without simulating the treatment process
136 at such. Therefore WWTP effluent was used as inoculum. It was sampled on the 5th of November
137 2019 from the Lynetten WWTP (Copenhagen, Denmark) which treats 57 million m^3/year. Lynet-
138 ten mainly treats domestic wastewater and the effluent is not disinfected prior to discharge to the
139 sea (Biofos WWTP, 2013, 2018). The sample was transported in an insulated bag and then stored
140 at 15 °C. The experiment was started within 8 h after sampling, without any further treatment of
141 the effluent sample.

142 Effluent temperature was 16.2 °C and the pH was 7.4, measured at the WWTP. A heterotrophic
143 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
144 h incubation at 20°C respectively on R2A agar (DS/EN ISO 8199). This heterotrophic plate count
145 is comparable to those found in lake and stream-water in an earlier study (Birch *et al.*, 2017b). The
146 three most abundant genera in the WWTP effluent, based on DNA sequencing, were the *12up*
147 belonging to the Betaproteobacteria class, and *Candidatus Planktoluna* and *Candidatus Rhodo-*
148 *luna* belonging to the Actinobacteria class (DNA sequencing methodology and results are provided

149 in SI 1, Table S1-S3). Further sample characterization included total suspended solids, non-volatile
150 organic carbon and conductivity (see SI 2, Table S4).

151 *2.3. Biodegradation testing*

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

162 The test systems were prepared in 20 mL gas tight vials in amber glass (Mikrolab Aarhus, Den-
163 mark) with magnetic screw caps and a silicone/PTFE septum (Sigma-Aldrich, Denmark). Biotic
164 test systems were prepared by adding 10 mL WWTP effluent to the vials and spiking with 1 μL
165 stock solution of cedarwood oil in methanol to a final cedarwood oil concentration of 57 μg/L.
166 Abiotic test systems were prepared in the same way, but using ultrapure water instead of the
167 WWTP effluent. The tested concentration of cedarwood oil corresponds to 0.01-15.2% of the es-
168 timated solubility of the main constituents, thus all main constituents were dissolved in the test
169 system (see SI 3, Table S5). The test systems were spiked using a gas tight 'eVol XR' automatic
170 syringe (SGE Analytical Science, Australia) and were closed immediately after spiking. Solvent

171 spiking was chosen as dosing method in order to transfer a defined mass to the test systems while
172 conserving the composition of the cedarwood oil. Initial tests with 0.03% methanol (vol:vol) and
173 25% headspace resulted in oxygen depletion and anaerobic conditions during the course of the
174 experiment (results not presented). The oxygen demand of the methanol was therefore estimated
175 before the next experiment and the headspace was dimensioned to provide a sufficient oxygen
176 reservoir (see SI 6). With a solvent addition minimized to 0.01% methanol (vol:vol), a 50% head-
177 space was deemed sufficient. The aerobic conditions were confirmed by oxygen measurements
178 throughout the experiment. The test systems were incubated at 12 ± 0.5 °C on tube roller mixers
179 at ~30 rounds per minute for the duration of the biodegradation experiments (up to 29 days).

180 *2.4. Chemical analysis*

181 After incubation the test systems were moved to a PAL RTC 120 autosampler (CTC, Zwingen,
182 Switzerland) for analysis without any manual sample preparation steps. Automated solid phase
183 microextraction (SPME) was performed using a 7 µm bonded polydimethylsiloxane (PDMS) fiber
184 (Supelco, USA). Separation and detection of the cedarwood oil constituents were done by Gas
185 Chromatography (GC) coupled to triple quadrupole Mass Spectrometry (MS/MS) (7890B GC sys-
186 tem / 7010B GC/TQ, Agilent Technologies, Denmark). The GC column (Agilent Technologies
187 122-5562 UI) was 60 m long with a 250 µm inner diameter and 0.25 µm film thickness and helium
188 was used as carrier gas at 1.2 mL/min. In the first biodegradation experiment, the test systems were
189 analysed with a Multiple Reaction Monitoring (MRM) method. In the second biodegradation ex-
190 periment, the test systems were analysed with a scan (MS2 scan) method. Details on the SPME-
191 GC-MS/MS methods are provided in SI 4.

192 *2.5. Identification of main constituents in cedarwood Virginia oil*

193 Prior to the biodegradation tests, the main constituents in the cedarwood Virginia oil were analysed
194 using SPME-GC and MS/MS in scan mode (method described in SI 4). Compounds were identi-
195 fied from the obtained ion spectra using spectral library match search in NIST (17) in the software
196 "Agilent MassHunter Unknown Analysis" after deconvolution. The fraction of each of the main
197 constituents in the cedarwood oil was determined by liquid injection of a dilution of the cedarwood
198 oil in hexane using GC coupled to a flame ionization detector (FID) (method description in SI 5).
199 The results of identification and fraction of main constituents were compared to the composition
200 analysis on the same batch of cedarwood oil provided by Givaudan, where a similar analytical
201 approach had been used.

202 In the present study it was possible to detect and identify eight main constituents in the cedarwood
203 Virginia oil: α-cedrene, cedrol, thujopsene, β-cedrene, β-chamigrene, β-himachalene, cuparene
204 and widdrol (ion spectra compared to spectra library match provided in SI 5, Figure S1). These
205 identifications were confirmed by the analysis from Givaudan. The fraction analyses were also
206 comparable (see SI 5, Table S8), but the analysis from Givaudan was based on a longer GC pro-
207 gram and therefore the constituents were separated better and the fraction analysis was more ac-
208 curate. Throughout this study, the fraction estimations of the main constituents provided by Givau-
209 dan were used (presented in Table 1). The present study covers all constituents in the tested batch
210 of cedarwood Virginia oil with a fraction >1%. The exception was β-funebrene with a fraction of
211 2% (see SI 5, Table S8), which could not be separated from α-cedrene in the MS/MS analysis. The
212 molecular structure of the eight main constituents and their physical- and chemical properties are
213 presented in Table 1.

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. (*1, *2)	Fraction in Cedarwood Virginia oil (%) (*2)	Molecular structure	Chemical formula (*1)	Molecular weight (g/mol) (*1)	Water solubility from K_{ow} (mg/L) (*3)	Log (K_{ow}) (*3)
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

*1 U.S. National Library of Medicine, 2019
*2 From composition analysis made by Givaudan
*3 National Food Institute DTU, 2019

219 *2.6. Data treatment: Main constituents*

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

225 $$PA_{relative} = \frac{PA_{biotic}}{PA_{abiotic}} \quad (1)$$

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

229 $$PA_{relative}(t) = \begin{Bmatrix} 1 & for\ t < t_{lag} \\ e^{-k_{system}(t-t_{lag})} & for\ t \geq t_{lag} \end{Bmatrix} \quad (2)$$

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

231 $$T_{\frac{1}{2},system} = \frac{\ln(2)}{k_{system}} \quad (3)$$

232

233 *2.7. Data treatment: Minor constituents and degradation products*

234 For each measuring day, triplicate Total Ion Chromatograms (TIC) from the scan method of biotic
235 test systems were compared to triplicate TICs of abiotic test systems in the software 'Agilent Mas-
236 sHunter Qualitative Analysis Navigator' by overlay of the chromatograms. In the retention time
237 (RT) interval where cedarwood oil constituents were present, 18.5 - 21.0 minutes, the heights of
238 minor peaks were compared, in order to investigate if minor constituents were biodegraded during
239 the course of the biodegradation test. Furthermore, it was investigated if new peaks or an increase

240 of existing peaks could be observed in the entire chromatogram of the biotic test systems, which
241 could be caused by formation of non-polar biodegradation products.

242 *2.8. Quality Assurance*

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

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

253 **Temperature and oxygen.** Test systems were incubated at 12 °C in a Binder KB 400 cooling
254 incubator (Binder, Germany). The temperature in the incubator was confirmed throughout the ex-
255 periment by an Easylog USB temperature datalogger, which showed only transient temperature
256 variations of 0.5-1 °C due to opening the incubator when vials were collected for analysis (see SI
257 6, Figure S2). The oxygen content in the test systems was measured at set time intervals with a
258 PreSens oxygen meter (Microx 4, Germany) using two different methods: a needle sensor and
259 contactless oxygen sensor spots. The initial oxygen concentration was 10.5 mg/L (20 °C). This
260 concentration was stable in the abiotic test systems throughout the experiment, whereas in the
261 biotic test systems it decreased during the first 14 days to a concentration around 7 mg/L at which

262 the concentration became stable. Throughout the experiment there were thus aerobic conditions in
263 both abiotic and biotic test systems (see SI 6, Figure S2).

264 **Calibration series.** Calibration series corresponding to 3.3%, 10%, 30% and 100% of the initial
265 spiked concentration in the test systems were analysed each measuring day, before and after the
266 series of triplicate biotic and abiotic test systems. The calibrants were prepared in 20 mL vials with
267 10 mL ultrapure water spiked with 1 μL stock solution of cedarwood Virginia oil in methanol. On
268 day 1, 3, 6, 9, 14, 22 and 28 the calibrants were analysed with the targeted analytical method
269 (SPME-GC-MS/MS MRM method, described in SI 4). On day 15 and 29 the calibrants were ana-
270 lysed with the non-targeted analytical method (SPME-GC-MS/MS scan method, described in SI
271 4).

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

279 **Quantification limit**. The lowest calibrant, 3.3%, was used as quantification limit, and thus rela-
280 tive peak areas below 0.033 were assigned the value 0.033. Due to decreasing instrument sensitiv-
281 ity for widdrol on day 22 and 28, no peak was observed for the 3.3% calibrant, the 10% calibrant
282 and for two out of the three biotic test systems on both days. Since a peak was detected for the
283 30% calibrant, biotic test systems with no peak observed are known to have less than 30% of the

284 initial spiked concentration left. When no peak was observed for widdrol in a biotic test system,
285 the relative peak area was therefore assigned the value 0.1 on those two days.

286 **Correction for abiotic losses.** Abiotic losses of the main constituents were determined based on
287 constituent specific peak areas in the abiotic test systems (n=3) relative to freshly spiked vials
288 (n=2). The highest loss was observed for β-himachalene, where the concentration in the abiotic
289 test system decreased 74% during the experiment. For α-cedrene, β-cedrene, thujopsene, β-chami-
290 grene and cuparene losses were 25-55%. No abiotic losses were observed for the more polar con-
291 stituents, widdrol and cedrol (results of abiotic losses are provided in SI 6, Figure S5). The ob-
292 served losses can be due to diffusion through the septum, sorption to test system or abiotic trans-
293 formation processes (Nagahama and Tazaki, 1987). Such abiotic losses should be kept at a mini-
294 mum and were in the present study corrected for by using the peak area ratio between biotic test
295 system and abiotic controls (Eq. 1).

296 *2.9. Bioaccumulation potential and baseline toxicity potential calculations*

297 It was investigated how the primary biodegradation of main constituents in the water phase of the
298 test systems impacted the bioaccumulation potential and the baseline toxicity potential of the mix-
299 ture. These calculations reflect the equilibrium partitioning of the main cedarwood oil constituents
300 into the lipid of an aquatic organism (bioaccumulation potential) and into the membrane of an
301 aquatic organism (baseline toxicity potential). The possible biotransformation in the organism was
302 thus not accounted for.

303 The initial concentration of a constituent, i, in the water phase, $C_{water,i,0}$, of the test system was
304 calculated from Eq. 5.

305
$$C_{water,i,0} = \frac{m_{cw} \cdot f_i \cdot f_{water,i}}{V_{water}} \quad (5)$$

306 Where m_{cw} is the mass of cedarwood oil added to the test system, f_i is the fraction of constituent i

307 in the cedarwood oil (Table 1), V_{water} is the volume of water in the test systems and $f_{water,i}$ is the

308 fraction of the constituent i in the water phase calculated from partitioning between headspace and

309 water phase Eq. 6.

310 $$f_{water,i} = \frac{1}{1+K_{air/water,i} \cdot \frac{V_{air}}{V_{water}}} \quad (6)$$

311 $K_{air/water,i}$ is the air-water partitioning coefficient of the constituent i (see Table 2), and V_{air} is the

312 volume of air in the test system.

313 The initial concentration of constituent i in water was converted to the corresponding equilibrium

314 concentration in storage lipid, for bioaccumulation potential estimations, and in membrane lipid,

315 for baseline toxicity potential estimations by multiplying with the respective lipid-water, $K_{lipid/water}$,

316 and membrane-water, $K_{membrane/water}$, partitioning coefficients. Partition coefficients from the UFZ-

317 LSER database are presented in Table 2 (Ulrich et al., 2017; Goss, 2019). The change in concen-

318 tration of a constituent in the lipid and membrane over time caused by biodegradation, $C(t)$, were

319 found using the average of the triplicate relative peak areas, $\overline{PA_{relative,i}}$, at the measuring day, t,

320 according to Eq. 7 and Eq. 8 .

321 $$C_{lipid,i}(t) = C_{water,i,0} \cdot K_{lipid/water,i} \cdot \overline{PA_{relative,i}}(t) \quad (7)$$

322 $$C_{membrane,i}(t) = C_{water,i,0} \cdot K_{membrane/water,i} \cdot \overline{PA_{relative,i}}(t) \quad (8)$$

323 The bioaccumulation potential and baseline toxicity potential of the cedarwood oil mixture (disre-

324 garding minor constituents and degradation products) were then estimated as the sum concentra-

325 tion of the main constituents in storage lipid and membrane lipid respectively at each measuring

326 day. The baseline toxicity was calculated in the unit mmol/kg assuming a membrane density of 0.9

327 kg/L, and the bioaccumulation potential was calculated in the unit g/kg assuming a lipid density
328 of 0.9 kg/L. The results of the calculated concentration of the cedarwood oil mixture (disregarding
329 minor constituents and degradation products) in lipid and membrane were plotted using GraphPad
330 Prism v.8.

331 **Table 2.** Air-water, $K_{air/water}$, membrane-water, $K_{membrane/water}$, and lipid-water, $K_{lipid/water}$ partitioning coefficients
332 modelled by the UFZ LSER Database for the eight main constituents in the tested cedarwood Virginia oil. RMSE
333 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	$9.8 \cdot 10^4$	$1.4 \cdot 10^5$

334

335 ## 3. Results and discussion

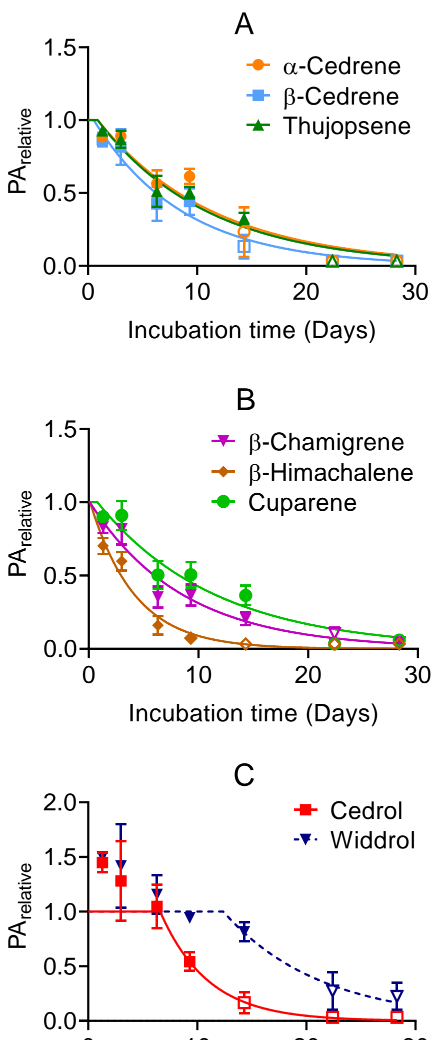
336 ### *3.1. Primary biodegradation kinetics of main constituents*

337 A first order degradation model with lag phase fitted to experimental biodegradation data for each
338 of the eight main constituents in cedarwood Virginia oil are shown in Figure 1. More than 90%
339 primary biodegradation was observed within the 28 day test duration for the constituents: α-
340 cedrene, β-cedrene, thujopsene, β-chamigrene, β-himachalene, cuparene and cedrol. More than
341 50% primary biodegradation was seen in all replicates on day 28 for widdrol. Widdrol may have

342 been degraded even more, but a more exact extent of degradation could not be quantified due to a
343 decrease in instrument sensitivity on the MS/MS on day 22 and 28. Widdrol had the lowest re-
344 sponse of the main constituents because widdrol only constitutes ~2% of cedarwood oil and is
345 more polar leading to lower enrichment during SPME extraction. The first-order degradation rate
346 constants were found to be within the same order of magnitude for all eight main constituents,
347 0.09-0.25 d^{-1}, resulting in test system half-lives of 2.8-7.5 days (see SI 7, Table S14). Constituents
348 with air-water partitioning coefficients >1 L/L have much higher first order biodegradation rate
349 constants in the water phase than determined for the test systems because the headspace acts as a
350 buffer replenishing the water phase when degradation occurs (Birch *et al.*, 2017a). Test system
351 biodegradation rate constants were converted to water phase biodegradation rate constants using
352 air-water partitioning coefficients according to Birch et al. (2017a) (see SI 8). This conversion led
353 to markedly higher rate constants for α-cedrene and β-cedrene, which however is associated with
354 some uncertainty related to the estimation of partition coefficients. To our knowledge, biodegra-
355 dation kinetics data have not previously been published for cedarwood oil or its constituents, but
356 previous studies have investigated their ready biodegradability (ECHA, 2011, 2018; Jenner *et al.*,
357 2011). The results of the previous published studies are in agreement with the findings in the pre-
358 sent study, that main cedarwood oil constituents are biodegradable (see SI 9).

359 In the present study α-cedrene, β-cedrene, thujopsene, β-chamigrene, β-himachalene and cuparene
360 had a short lag-phase < 1 day (see Figure 1). The short lag-phase indicates that bacteria able to
361 degrade these constituents were present in sufficient numbers in the effluent from the WWTP.
362 Bacterial growth and adaptation were therefore not necessary for initiating the biodegradation of
363 these cedarwood oil constituents. Widdrol had a lag-phase of 12.4 days and cedrol of 6.6 days.
364 The model fit of the lag-phase was poor for these two constituents, since the response in the biotic

365 test systems was 1.5 times higher compared to the response in the abiotic test systems after one
366 day of incubation, where it was expected that the response from biotic and abiotic test systems
367 would be equal. We have found no explanation for this observation.

368 

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

373

374 *3.2. Biodegradation of minor constituents and degradation products*

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

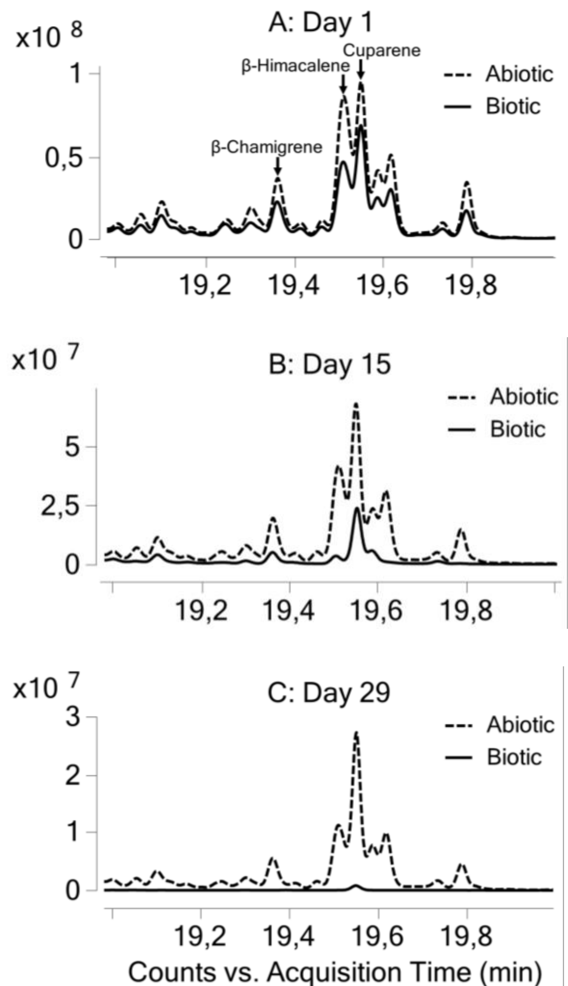


Figure 2. Biodegradation scan results for minor constituents in the tested cedarwood oil. A sub-section of the full chromatogram chosen to illustrate the disappearance of the minor peaks. Three of the peaks are included in the MRM method (β-chamigrene, β-himachalene and cuparene), the remaining peaks 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).

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

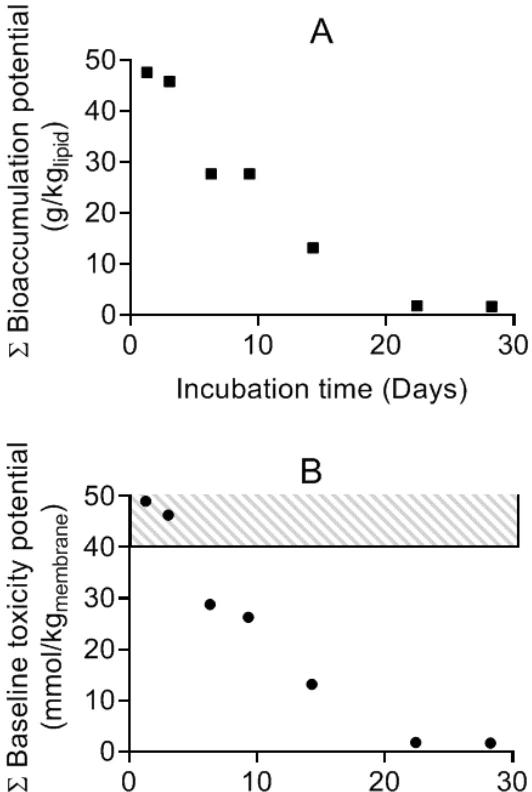
The initial contribution of each constituent to baseline toxicity and bioaccumulation potential was evaluated from their air/water, membrane/water and lipid/water partition coefficients (Table 2) and from the initial fraction of each constituent (Table 1). The constituents with the calculated highest 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 Figure 3 was similar to the biodegradation curves of these constituents in Figure 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 Figure 3B). This level was not seen to prevent the microorganisms in the test systems from degrading the cedarwood oil (Figure 1). Particularly the general

421 absence of significant lag-phases suggests that the test concentration was below an inhibitory level

422 (Hammershøj *et al.*, 2019). Reduced sensitivity to baseline toxicity in degrader microorganisms

423 compared to larger organisms have been reported earlier (Winding *et al.*, 2019).



424

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

428 *3.4. Improvements of UVCB testing and assessment*

429 The testing method suggested in the present study deals with several major challenges of whole

430 substance testing and provides an improved approach for testing biodegradation of UVCBs. There

431 are several advantages of whole UVCB substance testing combined with a targeted Multiple Re-

432 action Monitoring (MRM) and a non-targeted scan method. Constituent specific primary biodeg-

433 radation 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

456 degraded. Biodegradation kinetics of constituents could also be obtained from non-targeted anal-
457 ysis methods, which has recently been done covering 104 diesel oil constituents during whole
458 substance testing (Hammershøj *et al.*, 2020b). Identifying all constituents of very complex UVCBs
459 prior to testing can be very time consuming and tedious work. In this case it can be further inves-
460 tigated whether it is practical and sound assessing biodegradation of minor constituents by first
461 conducting a whole substance biodegradation experiment and at the end identifying those constit-
462 uents that did not degrade.

463 A limitation of the targeted analytical method is that only primary biodegradation is assessed. The
464 present study attempted to address this by investigating occurrence of non-polar degradation prod-
465 ucts with the scan method, but none were observed. It is possible that the degradation products
466 were too polar to be detected by SPME-GC-MS/MS or that degradation products are transient
467 because the constituents are completely mineralized. Since degradation products can be persistent
468 and toxic, these need to be considered as well, which for the case of cedarwood oil would require
469 additional analysis, such as LC-MS, that covers more polar compounds. Polar substances are usu-
470 ally less bioaccumulative, contribute less to baseline toxicity but can still have a specific mode of
471 toxic action. The method used in this study could be further developed, e.g. by applying different
472 analytical platforms to an assessment of what is remaining at the end of the biodegradation exper-
473 iment. If any degradation products are found, a screening of their bioaccumulation and toxicity
474 properties could be done in order to evaluate if further testing is needed.

475 4. Conclusion

476 This study introduced an improved biodegradation testing method for UVCBs by combining whole
477 substance testing with a targeted analytical method. The method was tested on cedarwood Virginia

478 oil, where primary biodegradation kinetics were successfully obtained for eight main constituents:
479 α-cedrene (23%), cedrol (21%), thujopsene (19%) β-cedrene (5%), β-chamigrene (1%), β-hima-
480 chalene (2%), cuparene (1%) and widdrol (2%). First-order degradation rate constants of these
481 main constituents ranged between 0.09 and 0.25 d^{-1}. The non-targeted GC-MS/MS analysis
482 showed biodegradation of many minor constituents. For some UVCBs it will be possible to apply
483 a more comprehensive analytical approach, which then can yield primary biodegradation kinetics
484 of more minor constituents. Due to the complexity of UVCBs, that can have many thousands of
485 constituents with varying physicochemical and fate properties, testing on the whole substance will
486 be advantageous for more efficiently obtaining data for UVCBs and constituents while accounting
487 for mixture effects. Additionally, time resolved bioaccumulation and baseline toxicity potential of
488 UVCBs can be estimated from primary biodegradation kinetics and partitioning coefficients,
489 where both whole substance effects and the difference in fate properties between constituents are
490 accounted for. For cedarwood oil, the primary biodegradation of main constituents resulted in a
491 substantial decrease of the bioaccumulation potential and baseline toxicity potential. Further re-
492 search should be directed to cover a broader range of UVCBs and UVCB constituents, and also
493 biodegradation products, which likely will require new combinations of sampling and analytical
494 methods.

495 **Declarations of interest**

496 None.

497 **Supporting Information**

498 The following files are available: DNA sequencing, Characterization of WWTP effluent inoculum,
499 Tested concentration of cedarwood oil constituents compared to solubility, Analytical methods,
500 Identification of cedarwood oil constituents, Quality Assurance, Cedarwood oil biodegradation
501 kinetics, Calculated biodegradation rate constants in the water phase, Published data on biodegra-
502 dation of cedarwood oil and Biodegradation chromatograms. This material is available free of
503 charge via the Internet.

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