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Whole UVCB tests can yield biotic and abiotic degradation kinetics of known and unknown constituents for an enhanced UVCB degradation profile

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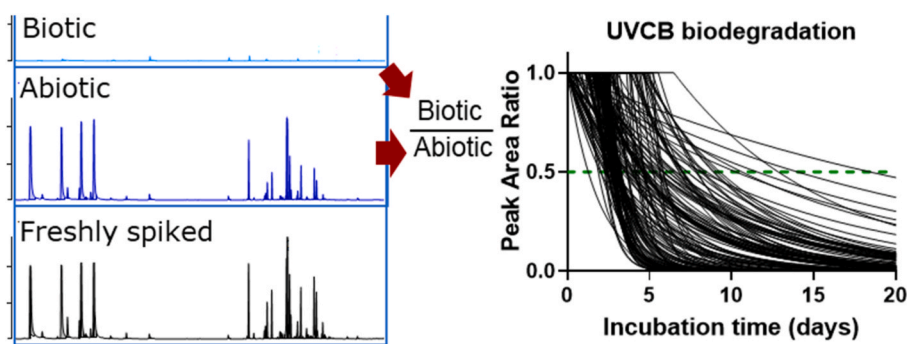
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HIGHLIGHTS

- Whole UVCB biodegradation tests were conducted with two essential oils.
- Degradation of 8–12 known and >78 unknown constituents were determined at ng-µg/L.
- Arrow Solid Phase Microextraction GC-MS provided biotic/abiotic peak area ratios.
- Fast biotic degradation (<20 days) was observed for all measured constituents.
- Native stream organisms were competent degraders for all measured constituents.

GRAPHICAL ABSTRACT



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ABSTRACT

The green transition and move towards safe and sustainable-by-design chemicals entail the need for new methods to study the biodegradability of UVCBs (substances of Unknown or Variable composition, Complex reaction products, and Biological materials). Standard simulation biodegradation tests have been developed for single substances and are generally not applicable for UVCBs. The aims of this study were (1) to combine a whole UVCB biodegradation test with a sensitive constituent-specific analytical technique, (2) to measure biotic and abiotic degradation of known and unknown UVCB constituents, and (3) to determine the impact of a wastewater treatment plant (WWTP) discharge on the constituent specific biodegradation in stream water. Lavender oil and black pepper oil are of significance in the perfume and cosmetics industries and served as model UVCBs. Stream water sampled upstream and downstream of a WWTP discharge point was characterized and used as inoculum (i. e., naturally and wastewater-adapted bacterial consortia). Tests were conducted in gastight headspace vials, and automated Arrow Solid Phase Microextraction GC-MS-scan was applied on unopened vials. Peak area ratios

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between biotic test systems and abiotic controls were used to determine primary biodegradation kinetics, and freshly spiked analytical references to separate biotic from abiotic degradation. Biodegradation half-times were below 20 days for all known (8–12) and unknown constituents (>78) in the essential oils. A dual-column GC-MS analysis produced a level 2 identification of 16 unknown lavender constituents. Biodegradation kinetics were similar in inoculum taken before and after the WWTP outlet, confirming that native stream microorganisms were competent degraders.

1. Introduction

A key element in the green transition and in various chemical regulatory frameworks (European Commission, COM, 2021) is the substitution of hazardous chemicals and the promotion of safe and sustainable-by-design chemicals (European Commission, COM, 2020). This requires environmental hazard and risk assessment approaches that also apply to complex substances of natural origin, which are categorized as UVCBs (substances of Unknown or Variable composition, Complex reaction products, and Biological materials) (Lai et al., 2022; Salvito et al., 2020, 2022). However, the persistence assessment of such UVCBs is still at a method development stage, and improved approaches for biodegradability and persistence testing of UVCBs are thus urgently needed (Lai et al., 2022; Birch et al., 2023; Davenport et al., 2022).

In European regulations, a substance is presently considered persistent if it contains one or more persistent constituents (for complex UVCBs, also groups of similar constituents) in concentrations >0.1% weight by weight (w/w) (European Chemicals Agency, 2023). Whole substance biodegradation tests that measure unspecific parameters (e.g., CO₂ evolution) reveal the properties of the whole substance but not the individual constituents. A UVCB may, therefore, contain persistent constituents (>0.1%), although the overall degradation is sufficient to meet the criteria for ready biodegradability (e.g., 60% theoretical CO₂ evolution) (European Chemicals Agency, 2023). Therefore, in many cases, a representative constituent approach is adopted, where structures are chosen and evaluated separately in mono-constituent biodegradation tests (European Chemicals Agency, 2023). It requires detailed knowledge of the composition of the UVCB to identify structures that fully represent the UVCB (Yordanova et al., 2021).

Persistence assessment is conducted through a tiered approach where the stringent lower tier screening tests for ready biodegradability, e.g., OECD 301 (OECD 301, 1992), can be used to categorize chemicals as not persistent, whereas only the higher tier simulation biodegradation tests, e.g., OECD 307-309¹¹, can be used to definitively determine persistence (half-lives >40 days in freshwater/estuarine water or > 60 days in marine water) (European Chemicals Agency, 2023). Recently, a simulation-type biodegradation testing approach was developed for testing hydrophobic and volatile chemicals in mixtures at environmentally relevant concentrations and using constituent specific analysis to determine primary biodegradation kinetics (Birch et al., 2018, 2023; Hammershøj et al., 2020; Møller et al., 2021, 2024). In the current study, we applied this approach for whole UVCB testing and combined it with sensitive analytical methods to provide enhanced biodegradation profiles of known and unknown UVCB constituents. The whole substance test ensures that all constituents are present in the test. The targeted analysis ensures the determination of primary biodegradation kinetics of known constituents, whereas the non-targeted analysis allows the determination of primary biodegradation kinetics of unknown constituents without prior identification.

Since essential oils are used in increasing amounts in consumer products, in the food industry (Saeed et al., 2022), and as biopesticides (Gupta et al., 2023), lavender and black pepper essential oils were used in this study as model UVCBs. Essential oils mainly contain terpenes and terpenoids, many of which are volatile or semi-volatile. A closed test system minimized evaporative losses during incubation, and fully automated Solid Phase Microextraction on unopened test systems was applied to avoid evaporative losses during sample preparation and

analysis. Primary biodegradation was determined from peak area ratios between biotic test systems and abiotic controls (Birch et al., 2018). The abiotic controls served as an experimental and analytical reference, which accounted for minor abiotic losses during incubation and for analytical instrument sensitivity variations. The abiotic controls enabled the determination of biodegradation kinetics for unknown constituents without prior identification (Birch et al., 2018, 2023; Hammershøj et al., 2020; Møller et al., 2021). A previous study showed a substantial decline of lavender oil constituents in abiotic controls (Hammershøj et al., 2020). This was best explained by abiotic degradation processes (e.g., hydrolysis, autooxidation). A freshly spiked analytical reference was therefore included in this study to separate and determine biotic and abiotic degradation or losses.

Biodegradation kinetics of chemicals can often be faster in pre-exposed environments than in pristine environments because of microbial adaptation (Poursat et al., 2019). Wastewater treatment plants are pre-exposed to essential oil constituents due to their use in consumer products, such as cleaning products, perfumes, or well-being products. While many natural streams are not pre-exposed to a specific essential oil, such as lavender oil or black pepper oil, most plant species produce secondary metabolites that are chemically closely related to each other. Some general metabolic pathways are shared within the plant realm to access platform molecules such as isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are transformed into higher-order analogs. Chemo diversity and specific chemical composition are attained with the interplay of other metabolic pathways in each plant species. As a result, essential oils' constituents vary both qualitatively and quantitatively from one plant species to another but frequently have in common simple terpenes (containing n C₅H₈ units ($n > 2$) formed by the combination of IPP and DMAPP subunits) and terpenoids (containing oxygen-containing functional groups) that are ubiquitous (e.g., *para*-cymene, pinenes, caryophyllene and so on). The low level of substrates in stream water forces microorganisms to grow on mixed substrates present at low concentrations (Egli, 2010). The presence of plant residues in streams could, therefore, lead to generic pre-exposure of microorganisms to terpenes and terpenoids. Wastewater treatment plant discharges to natural streams change the microbial consortia downstream of the discharge point through the discharge of microbes from the treatment plant (Mansfeldt et al., 2020; Desiante et al., 2022). In this study, inoculum sampled upstream and downstream of a local wastewater treatment plant was used to investigate the difference between biodegradation kinetics in naturally pre-exposed inoculum and wastewater treatment plant pre-exposed inoculum.

The aims of this study were (1) to combine a whole UVCB biodegradation test with a sensitive constituent-specific analytical technique, (2) to measure biotic and abiotic degradation of known and unknown UVCB constituents, and (3) to investigate how WWTP discharge impacts the constituent specific biodegradation in stream water. The hypothesis of the third aim was that microorganisms from the WWTP effluent are preadapted to multiple chemicals, including essential oils, which leads to faster degradation downstream of the WWTP discharge point compared to upstream.

2. Materials and methods

2.1. Materials

The test substances were Lavender Oil Barreme Type (CAS 8000-28-0) and Black Pepper Oil Madagascar Orpur (CAS 8006-82-4/84929-41-9) (Givaudan, UK). Their main constituents, according to the certificate of analysis from Givaudan, are shown in [Tables S1 and S2](#). Ultrapure water was produced using an Elga Purelab flex water system (Holm & Halby, Denmark).

2.2. Inoculum

Stream water was sampled from the Jydebæk stream (Zealand, DK) before and after the outlet from Holme Olstrup wastewater treatment plant (WWTP) on September 19th and October 18th, 2022. Upstream of Holme Olstrup, the Jydebæk stream is rather small and drains agricultural fields (see picture in [Fig. S1](#)). Holme Olstrup WWTP outlet is the first WWTP discharge point on this branch of the Suseåen stream, which is the largest stream in Zealand (DK). The Holme Olstrup WWTP is a medium-sized treatment plant that treats 6500 person-equivalents of wastewater. It has mechanical and biological treatment steps, nitrification, denitrification, and phosphorus removal. Samples were collected in glass bottles. The samples were transported to the lab in insulated bags, and the test setup was completed within 6 h of sampling. The sampling temperature was between 11.5 and 13.5 °C, and the incubation temperature of the experiment was 12 °C. Background characterization of the stream water inoculum in terms of temperature, oxygen (7.7–9.7 mg/L), pH (7.4–8.0), total suspended solids (3.8–5.0 mg/L), non-volatile organic carbon (6.4–8.7 mg/L), and microbial community analysis is shown in [Supporting Information S1 \(Table S3 and Fig. S2\)](#). Microbial community characterization was performed by DNASense, who analyzed the samples using gene amplicon sequencing targeting the bacteria 16S rRNA gene variable regions 1–8 (bV18-A) in combination with taxonomic classification against the SILVA 138.1 99 % NR database ([Supporting Information S2](#)). Raw sequencing reads are available on GenBank under Bioproject ID PRJNA1111435.

2.3. Biodegradation experiments

One biodegradation experiment was conducted with lavender oil (Sept 19th) and one with black pepper oil (Oct 18th). Each of these consisted of 21 test systems inoculated with surface water collected upstream of the WWTP outlet, 21 test systems inoculated with surface water collected downstream of the WWTP outlet, and 21 abiotic controls, for triplicate measurements at 7 time points. The lavender oil experiment was duplicated and analyzed using two analytical methods – a targeted method on a gas chromatograph with triple quadrupole mass spectrometer (GC-MS/MS) for the known constituents and a non-targeted gas chromatography-mass spectrometry (GC-MS) scan method for unknown constituents. The black pepper experiment was analyzed using a non-targeted scan method only, as the scan method had sufficient separation and sensitivity. In total, more than 150 test systems were prepared and analyzed.

2.4. Test concentration

Environmentally relevant test concentrations are defined as <1–100 µg/L in standard mono-constituent tests in surface water (OECD 309) (OECD 309, 2004), and a biodegradation study found that a mixture concentration of ~40 µg/L (0.5 µg/L per chemical) was more environmentally relevant than ~400 µg/L (5 µg/L per chemical) (Tian et al., 2023). Since the proportions of the constituents in UVCBs vary highly, lowering the test mixture concentration comes at the cost of fewer detectable unknown constituents. The initial whole UVCB concentration level in this study was therefore set at 150 µg/L, which corresponds to

concentrations of each constituent in the range of 30 ng/L to 60 µg/L (see [Table S6](#)). This concentration level was combined with a highly sensitive analytical extraction method, arrow solid phase micro-extraction (Kremser et al., 2016; Birch et al., 2022), to ensure the determination of biodegradation kinetics of as many constituents present in the essential oils as possible.

2.5. Preparation of test systems

Ultrapure water was chosen for abiotic controls to avoid the use of sodium azide for poisoning environmental samples, to minimize toxic waste generation, and because it is robust against biological re-growth during month-long incubation times. Partitioning of hydrophobic chemicals to dissolved organic carbon (DOC) and suspended solids (TSS) in stormwater runoff was in a previous study (Birch et al., 2012) seen to be limited at organic carbon levels corresponding to the inoculum in this study (<9 mg/L DOC, <5 mg/L TSS). In another study with hydrocarbons in the log octanol-water partition ratio range of 3–6, abiotic controls prepared in ultrapure water produced similar results as abiotic controls prepared in azide-sterilized river water (Sjøholm et al., 2021). Log octanol-water partition ratios listed in the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) for 7 of the 13 known constituents in lavender oil and 5 of 8 known constituents in black pepper oil span from 2.4 to 6.3. Sorption of the test chemicals to organic carbon and particulates during extraction with SPME for analysis is therefore not expected to impact the biodegradation results.

Test systems were prepared in 20 mL amber precision thread headspace vials (Microlab, Denmark). 15 mL inoculum (stream water) was added to biotic test systems, and 15 mL ultrapure water was added to abiotic test systems. Test systems were then spiked to reach the test concentration of 150 µg/L using 1 µL of a 1:1 ethanol:water solution containing lavender oil or an ethanol solution containing black pepper oil with a gas tight eVol XR automatic syringe (SGE Analytical Science, Australia). After spiking, test systems were immediately closed with magnetic screw caps with polytetrafluoroethylene (PTFE)/silicone septa (Sigma-Aldrich, US). Blanks were prepared with ultrapure water without essential oils.

Test systems were incubated at 12 °C on benchtop rollers, and at time points between 1 and 29 days, one blank, triplicate biotic, and triplicate abiotic test systems were moved to the autosampler for analysis (lavender was analyzed with targeted analysis on day 1, 4, 7, 10, 14, 21, and 28, scan analysis on day 2, 8, 15, 22, and 29, and black pepper was analyzed with scan analysis on day 3, 6, 9, 13, and 20). More test systems were prepared than analyzed. However, the test was stopped when peaks in biotic test systems were reduced to close to the blank signal. Triplicate freshly spiked controls were prepared like abiotic controls on each day of analysis and analyzed along with the incubated test systems alternating abiotic, biotic, and freshly spiked test systems. Samples were analyzed within 10 h of being placed on the autosampler (at room temperature).

2.6. Oxygen measurements

It is important to ensure that the test remains aerobic when using ethanol to spike a closed biodegradation test since the degradation of ethanol consumes oxygen. Aerobic conditions in the test vials were verified for each experiment and surface water by adding ½ or 1 µL ethanol to 15 mL surface water in 20 mL headspace vials fitted with optical oxygen sensor dots (Presens, Germany), incubating these vials in parallel with the other test vials, and measuring the oxygen at each sampling point. The addition of 1 µL ethanol resulted in oxygen concentrations between 4 and 6 mg/L from day 7–29, and the addition of ½ µL ethanol resulted in oxygen concentrations of 6–8 mg/L.

2.7. Chemical analysis

Automated extraction of analytes was obtained on a PAL RTC 120 autosampler (CTC Analytics AG, Switzerland) using immersion Arrow - Solid Phase Microextraction (Arrow-SPME) (Kremser et al., 2016) depletive sampling directly on the unopened test systems. The autosampler was coupled to a 7890B gas chromatograph (GC) with a triple quadrupole mass spectrometry detector (MS/MS) (7010B GC/TQ) (Agilent Technologies, US).

The targeted method for lavender was as follows. A polydimethylsiloxane (PDMS) Arrow-SPME fiber (diameter 1.1 mm, phase thickness 100 μm) was conditioned at 280 $^{\circ}\text{C}$ for 5 min, and the sample was heated to 35 $^{\circ}\text{C}$ for 10 min. 15 min extraction was done at 35 $^{\circ}\text{C}$ with agitation of 250 rpm 5s on 2s off, and the Arrow-SPME fiber immersed in the liquid. Analytes were desorbed in the GC inlet at 280 $^{\circ}\text{C}$ for 10 min. The GC was fitted with a 60 m \times 250 μm \times 0.25 μm DB-5ms Ultra Inert column (Agilent Technologies, US), and helium was used as carrier gas (1.2 mL/min). The GC oven temperature was held for 10 min at 50 $^{\circ}\text{C}$ during desorption, then the temperature was ramped at 10 $^{\circ}\text{C}/\text{min}$ to 290 $^{\circ}\text{C}$. Detection was performed using a triple quadrupole detector (MS/MS) in multiple reaction monitoring mode (MRM). The MRM transitions used for quantification of the known constituents of lavender oil are listed in Table S2. The non-targeted method for lavender was done as described above, except that the GC oven temperature was ramped at 5 $^{\circ}\text{C}/\text{min}$ to 200 $^{\circ}\text{C}$ followed by 30 $^{\circ}\text{C}/\text{min}$ to 290 $^{\circ}\text{C}$. This lower ramp was chosen to get a slightly better separation of minor peaks in the non-targeted analysis. Detection in the non-targeted analysis was performed with MS scan from 40 to 400 m/z . The black pepper method was similar to the non-targeted method for lavender, except that the extraction time was set to 5 min.

2.8. Data analysis

Known constituents in lavender oil were quantified through MRM analysis and integrated using the QQQ Quantitative Analysis software (Agilent Technologies, US). Known constituents in black pepper oil were quantified by selected target ions in the scan chromatograms (Table S4) and integrated with the MS Quantitative Analysis software (Agilent Technologies, US).

The non-target datasets were integrated in MS-DIAL ver. 4.9.221218. Data files were first converted into ABF files by the Analysis Base File Converter (Reifycs Inc., Japan). Peak detection was done with a minimum peak height of 2000, and alignment was done with an EI similarity tolerance of 80% and a retention time tolerance of 0.075 min. Blank filtering was done with the criteria: the feature was removed if the peak area max in samples was less than 5 times the blank peak area average. All features were looked through manually and marked if the peaks were clearly defined and present in the freshly spiked controls. The marked peaks were then simultaneously integrated in all samples through the 'aligned chromatogram viewer for simultaneous curation'. Peak areas were imported to Excel for further filtering. Features were included if the average peak area in freshly spiked controls was larger than ten times a limit determined as blank signal + ten times standard deviation on the blank signal. This was done to focus data treatment on those features where biodegradation of >90% could be determined.

During the deconvolution of scan chromatograms for large peaks, some chemicals may split into more than one feature. Multiple chemicals may also appear at the same retention time. Therefore, the number of features does not correspond directly to the number of chemicals. A minimum number of non-targeted chemicals was therefore determined by choosing one feature for each retention time ± 0.01 min.

Peak area ratios were calculated between 1) upstream biotic test systems divided by abiotic test systems, $R_{\text{upstream biotic/abiotic}}$, 2) downstream biotic test systems divided by abiotic test systems, $R_{\text{downstream biotic/abiotic}}$, and 3) abiotic test systems divided by freshly spiked control, $R_{\text{abiotic/freshly spiked}}$. This was done for known constituents and the

selected features in the non-target analysis. These peak area ratios revealed 1) biodegradation in water upstream of the WWTP outlet, 2) biodegradation in water downstream of the WWTP outlet, and 3) abiotic losses or abiotic degradation.

The biotic/abiotic peak area ratios ($R_{\text{upstream biotic/abiotic}}$ and $R_{\text{downstream biotic/abiotic}}$) were used to determine biodegradation kinetics by fitting the ratios to a first-order degradation model with lag phase, t_{lag} , in GraphPad Prism 10.1.1. In the first-order degradation model (equation (1)), k is the first-order degradation rate constant, and t is the incubation time. A maximum of two peak area ratios were included if biotic test systems were below a limit set by the blank signal + ten times the standard deviation on the blank signal. These data points are indicated by open symbols in biodegradation plots. First-order half-lives, $t_{1/2}$, were calculated as $\ln(2)/k$, and degradation half-times, DegT_{50} , as the sum of the first-order half-life and the lag phase.

$$R_{\text{biotic/abiotic}} = \begin{cases} 1 & \text{for } t < t_{\text{lag}} \\ e^{-k(t-t_{\text{lag}})} & \text{for } t > t_{\text{lag}} \end{cases} \quad (1)$$

2.9. Quality assurance

Linearity of the signal was confirmed for known constituents from standard curves covering 5–150 $\mu\text{g}/\text{L}$ of the essential oils. The constituent β -caryophyllene showed non-linearity over this range of concentrations in both lavender and black pepper standard curves (higher concentrations showing lower signal than expected from linearity). It is speculated that it may be caused by this chemical being spiked above its solubility since the peak is not higher than other peaks in the chromatogram. In black pepper experiments, δ -3-carene and limonene showed slight non-linearity at the highest concentration because of overshoot of the MS. This curvature leads to slight conservatism in biodegradation data. 3-octanone was only detectable at 45–150 $\mu\text{g}/\text{L}$ in lavender oil.

Analytical (SPME fiber) carryover was evaluated in the black pepper experiment from 6 blank test systems run after freshly spiked abiotic controls. Carryover on all known constituents were <0.1%.

The freshly spiked controls on day 21 for lavender oil were removed from the data set due to a faulty syringe, and abiotic losses are therefore not evaluated on this day.

2.10. Additional identification of lavender oil constituents

Level 2 identification (Schymanski et al., 2014) (i.e., identification of probable structure) of some of the unknown constituents in the lavender essential oil was obtained through dual column (polar and apolar) gas chromatography coupled to mass spectrometry (GC-MS, Clarus 690 SQ8T MS from PerkinElmer, US) in a 1% dilution in ethyl acetate. Retention indices were calculated based on the injection of a standard mix of alkanes C7–C40 on both columns and compared to retention indices from WebBook NIST. Quantitative composition was obtained through gas chromatography coupled to a flame ionization detector (GC-FID, GC-2010 Pro instrument from Shimadzu, Japan). The details of the analytical method are listed in supporting information S3. The identification of lavender oil constituents was coupled to the biodegradation data through unknown analysis of freshly spiked controls in MassHunter Unknown Analysis version 10.2 coupled to NIST 2017 Mass Spectral Library (Agilent Technologies, US) and then matched to biodegradation information from MS-DIAL (see specifications in supporting information S3). The identification and compositional information were used to calculate test concentrations for lavender oil constituents (Table S6).

3. Results and discussion

3.1. Known constituents

Of the 13 constituents listed in the certificate of analysis for lavender oil (sum to 91.2%), only one of these (lavandulol, 0.5%) was not detected in our experiments. The automated arrow SPME with a PDMS coating concentrates chemicals based on hydrophobicity because of the higher partitioning into the fiber for chemicals with higher octanol-water partition ratios. The selectivity of the PDMS coating and the larger coating volume of arrow SPME ensured the detection of additional constituents even though they were present in the lavender oil at lower concentrations. All eight black pepper constituents listed in the certificate of analysis (sum to 84.9%) were detected in the experiment, and many additional peaks were seen in the chromatogram.

3.1.1. Biotic degradation

The known constituents in lavender oil biodegraded with a half-time below 10 days in stream water, and known black pepper oil constituents with a half-time below 20 days. Biodegradation curves and abiotic controls are shown for all known constituents in Fig. S3, biodegradation kinetics are listed in Tables S7 and S8, and examples of biodegradation curves for four lavender and black pepper known constituents are shown in Fig. 1.

3.1.2. Abiotic degradation and losses

The abiotic controls for the known constituents in lavender oil can be grouped into four categories 1) fast abiotic degradation (linalyl acetate), 2) abiotic formation (linalool and α -terpineol), 3) some abiotic losses (limonene, *trans*- β -ocimene, *cis*- β -ocimene, and β -caryophyllene), and 4) stable abiotic levels (3-octanone, 1,8-cineol, camphor, terpinene-4-ol). Abiotic degradation through hydrolysis of linalyl acetate was expected due to its ester group, and half-lives <24 h have previously been reported at elevated temperature (50 °C) (OECD, 2002) or low pH (pH 3)²⁹. Here, at 12 °C and pH 7.4–8, the hydrolysis half-lives in the abiotic

controls were 4.4 days. The determination of this hydrolysis rate was possible due to the analysis of freshly spiked controls at each measurement point. Linalool and α -terpineol have previously been found as the two major hydrolysis products of linalyl acetate, where α -terpineol can be formed through cyclization of linalool (Clark et al., 1989). These two lavender oil constituents were formed abiotically in this study. However, it could be seen that the abiotic formation did not lead to increased concentrations in biotic test systems, where biodegradation of all three constituents (linalyl acetate, linalool, and α -terpineol) was fast.

Four of the known constituents in the lavender oil (3-octanone, eucalyptol, camphor, terpinene-4-ol), and most of the known constituents in the black pepper oil (6 of 8 constituents) showed stable abiotic controls (<30% removal). Some abiotic removal (>30%) was seen for four known constituents in lavender oil (limonene, *trans*- β -ocimene, *cis*- β -ocimene, and β -caryophyllene) and two constituents of black pepper (δ -elemene and α -humulene). These losses could stem from autooxidation (de Groot, 2019). However, the abiotic removal was markedly slower than the fast biodegradation seen for these constituents.

3.2. Unknown constituents

A first overview of the unknown constituents in lavender and black pepper oil was gained by looking at chromatograms from the last day of analysis, day 29 and 20, respectively (see Fig. 2). A zoomed-in picture of the biotic test system is shown in supporting information in Figs. S5 and S6. The chromatograms clearly show that it is not only the known constituents of the essential oils that biodegrade, but the biodegradation also applies to the unknown constituents (compare biotic chromatogram with abiotic chromatogram). The biotic lavender oil test system on day 29 showed no peaks that were not found in the blank test system, and all visible constituents at the chosen concentration level were, therefore, fully biodegraded. Some small peaks, not seen in the blanks, resided in the biotic black pepper chromatogram on day 20 at retention times from 32.5 to 34.5 min (see Fig. S6).

Fig. 3 shows the biodegradation curves for all extracted features in

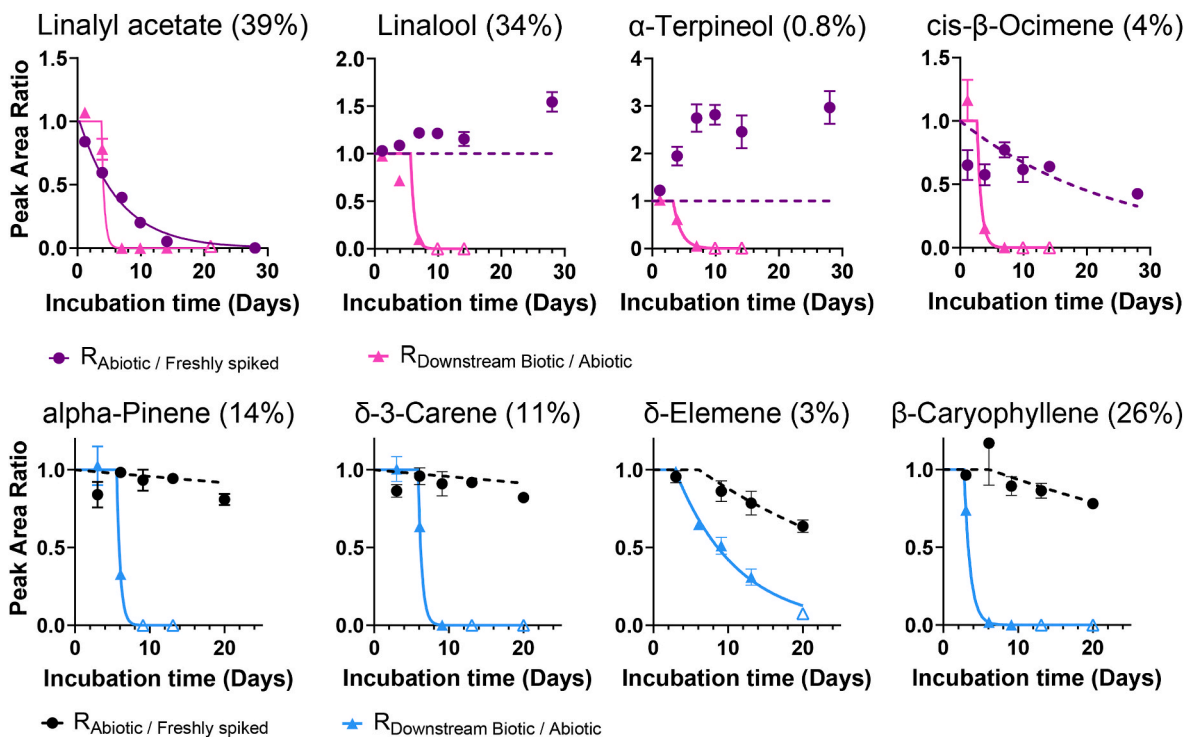


Fig. 1. Biodegradation in stream water sampled downstream of WWTP discharge point (triangles) and abiotic degradation/losses (circles) for four known constituents of lavender oil (top row) and black pepper oil (bottom row). The constituents were chosen for illustrative purposes. The percentage content of each constituent in the oil is indicated after its name. Error bars show the standard error of the mean.

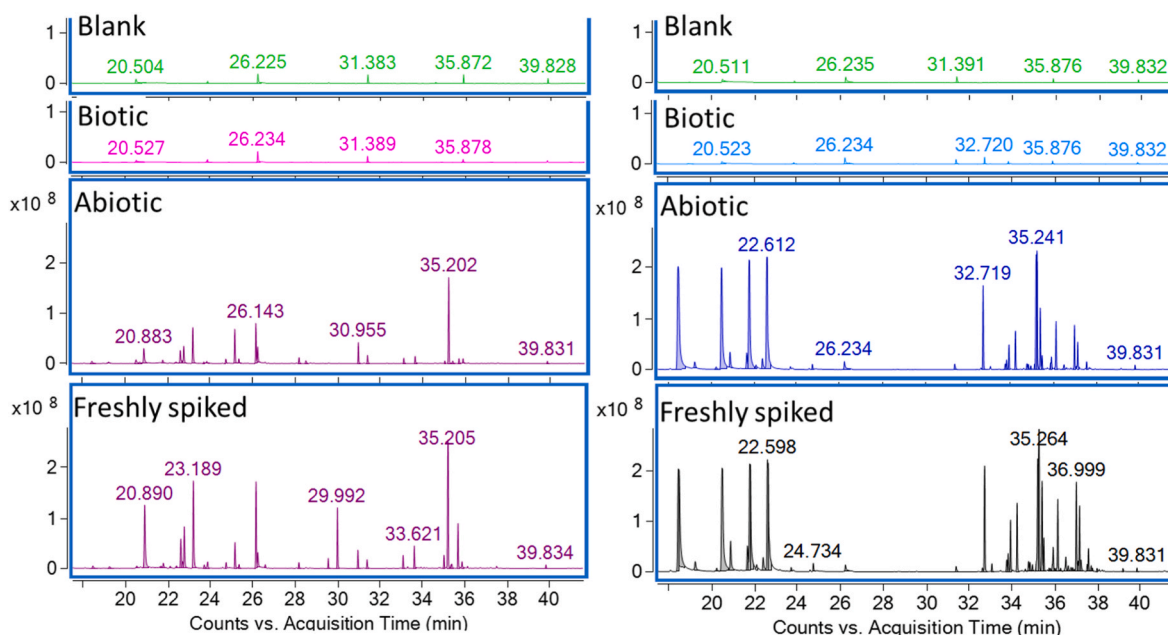


Fig. 2. Lavender (left) and black pepper (right) chromatograms after 29 and 20 days of incubation, respectively. Blank (top), biotic test system, abiotic test system, and freshly spiked control (bottom).

lavender and black pepper oil. It was possible to extract 132 features in lavender oil and 157 features in black pepper oil, which fulfilled the quality criteria described in the experimental section. These features corresponded to at least 78 and 80 different constituents (discrete retention times), respectively. The degradation curves showed that the features that resided in the black pepper chromatogram on day 20 were degraded >50% and thus did not stem from persistent constituents. Biodegradation half-times for the unknown constituents were below 11 days in lavender oil and below 20 days in black pepper oil in stream water upstream and downstream of the WWTP outlet. The biodegradation of these unknown constituents, therefore, aligned well with the biodegradation observed for the known constituents.

3.3. Additional identification of lavender oil constituents

The level 2 identification of lavender oil determined 97.0% of the composition of lavender oil based on FID. The 12 known constituents covered 89.4% of the UVCB in this analysis. Biodegradation data for an additional 16 level 2 identified constituents could be inferred from the dataset. These 16 constituents correspond to 4.23% of the mixture. Biodegradation curves for these constituents and corresponding biodegradation kinetics are shown in Fig. S8 and Table S9.

3.4. Natural versus wastewater-adapted inocula

The second aim of this study was to compare the effect of wastewater treatment plant pre-exposure to natural pre-exposure on the biodegradation kinetics of essential oils. This was done for known constituents and for discrete unknown features (retention times >0.01 min difference). The kinetics were evaluated based on biodegradation half-times, and downstream DT50 vs upstream DT50 is shown in Fig. 4. Similar plots for all features are shown in Fig. S7. Fig. 4 shows that while the known constituents in lavender oil degraded slightly faster in water sampled upstream than downstream of the WWTP, black pepper known constituents and unknown features were situated on both sides of the 1:1 line. Our hypothesis that biodegradation would be faster in stream water downstream of the WWTP was therefore not confirmed by the results. However, the results demonstrated instead that native stream water microorganisms were competent degraders for the essential oil

constituents. This observation strengthens the conclusion on the degradability of these essential oil constituents since it demonstrates the possibility of degradation, whether essential oils pass a wastewater treatment plant or are emitted directly into the environment.

3.5. Implications

The results from this study show that all the detected constituents in the two essential oils were degraded in the natural inoculum, and the UVCBs were, therefore, not found to be persistent. This result is generally in line with ready biodegradability tests on some of the single constituents found in the essential oils. In the European Chemicals Agency (ECHA's) registration dossiers, (European Chemicals Agency) the known constituents linalyl acetate, linalool, 1,8-cineole, camphor, terpinene-4-ol, α -terpineol, caryophyllene, α -pinene are registered as readily biodegradable. Half of the additionally identified lavender oil constituents are not registered as pure substances or lack biodegradation data in ECHA's dossiers. The second half of the additionally identified lavender oil constituents are registered: β -pinene, octen-3-ol, β -myrcene, hexyl acetate, γ -terpinene, terpinolene, linalool oxide, borneol, hexyl butanoate, bornyl acetate, and nerol acetate, are registered as readily biodegradable, farnesene and *cis*- β -bisabolene as not readily biodegradable, and (–)-camphene as persistent. However, additional studies show that (–)-camphene has passed a ready biodegradability test (Api et al., 2023), and at least one passed ready biodegradability test has been published for *D*-limonene (Api et al., 2022a), β -thujene (Api et al., 2022b), 1-octen-3-ol acetate (Api et al., 2019), borneol (Api et al., 2015), and α -humulene (Jenner et al., 2011).

The biodegradation tests conducted for the essential oil constituents have mainly been ready biodegradability tests (according to the OECD 301 or 310 guidelines) and, in a few cases, inherent biodegradation tests (according to the OECD 302 guideline). Ready biodegradability tests conducted at high concentrations with mono-constituents are, however, fundamentally different from the primary biodegradation simulation tests conducted at low concentrations in complex mixtures in the current study. While the high concentrations necessary for the ready biodegradability tests can lead to slower biodegradation due to the low solubility of essential oils or due to inhibition of the inoculum, these issues are avoided in the simulation biodegradation type tests. A limitation of

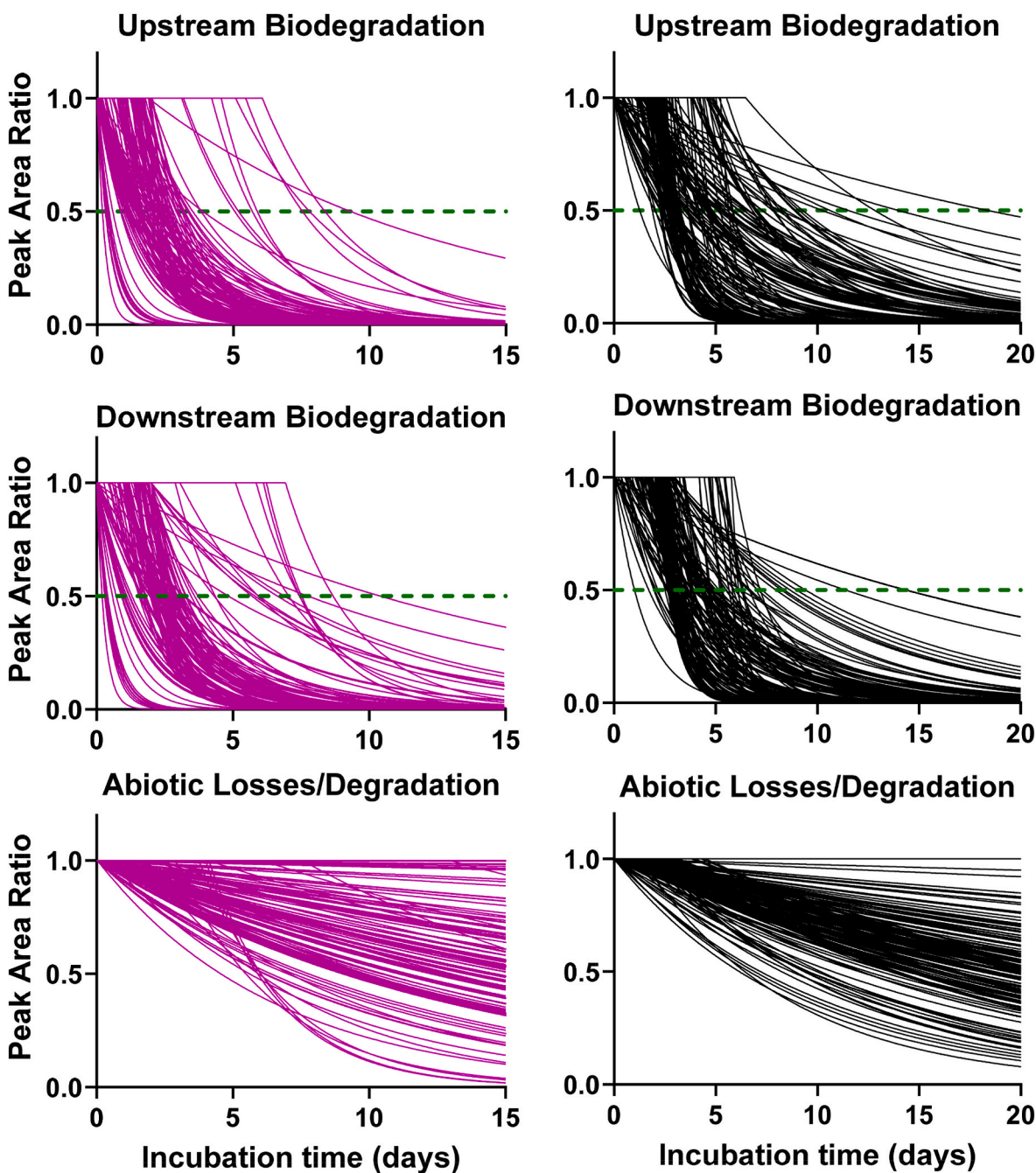


Fig. 3. Biodegradation curves based on unknown analysis of 5 data points for 132 features in lavender oil (left) (78 different retention times) and 157 features in black pepper oil (right) (80 different retention times). Biodegradation in water sampled upstream of the WWTP outlet (top) and downstream of the WWTP outlet (middle), and abiotic removal in abiotic controls (bottom). Green dotted lines illustrate 50% degradation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the simulation type test is that UVCBs of natural origin generally cannot be ^{14}C labeled. ^{14}C labeling of main constituents for use in single constituent tests could potentially reveal information on metabolites. The main information gained from whole UVCB simulation tests is, however, the primary degradation of the constituents. While the non-targeted analytical method used in this study (GC-MS) is suitable to evaluate whether substantial amounts of non-polar metabolites are formed, many metabolites are polar, and additional non-targeted LC-MS analysis would be needed to further investigate metabolites.

Unknown UVCB constituents are generally poorly addressed in persistence assessments of UVCBs, which often focus on selected known and representative constituents. One possibility for investigating the

knowledge gap of unknown constituents is to use *in silico* methods. In one study, the physical-chemical space of a UVCB was defined to generate sets of physical-chemical properties for hypothetical constituents which were then used for risk assessment of the UVCB (Sühling et al., 2022). In another study, hypothetical chemical structures were generated for UVCBs and run through QSAR models to gain information for hazard assessment of the UVCBs (Dimitrov et al., 2015). A prerequisite for these *in silico* methods is that boundaries for the unknown chemical structures and their properties can be estimated (Sühling et al., 2022). This implies that reliable data exists for a set of known constituents. The present study clearly shows how it is possible, with a carefully designed experiment, to investigate the biodegradation of UVCBs in

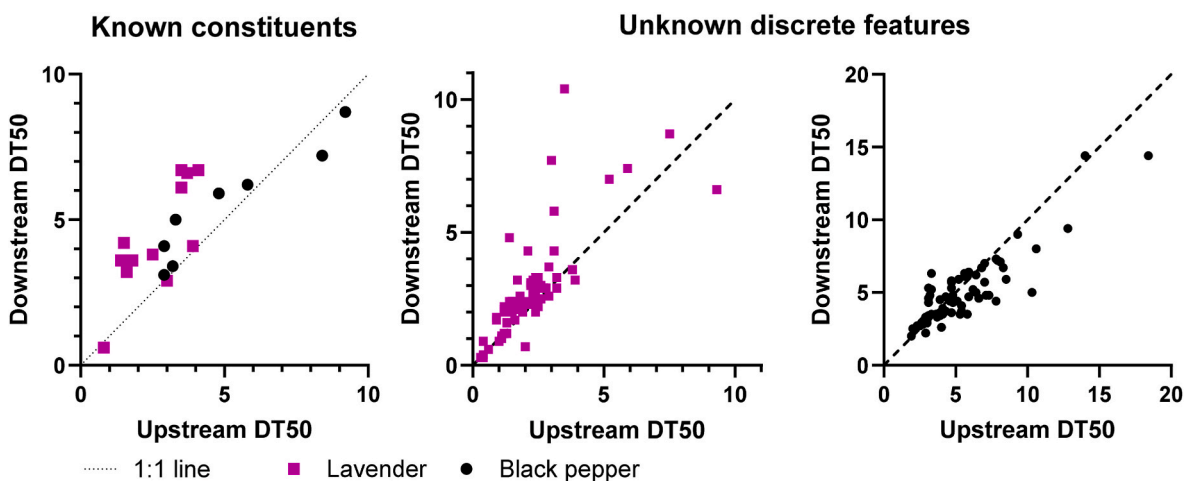


Fig. 4. Upstream and downstream half-times (DT50, days) of known lavender and black pepper constituents (left), discrete unknown features in lavender oil (middle) and black pepper (right).

whole substance experiments while determining the primary biodegradation of an enlarged range of mixture constituents by unknown analysis. This improved overview of biodegradation properties of mixture constituents is a huge advantage in the assessment of UVCBs because biodegradation testing is not limited by the need for detailed characterization of the UVCB. These experimental investigations of biodegradation of the unknown mixture constituents can, therefore, complement *in silico* methods for risk assessment of UVCBs. When instrumentation is available for an increased characterization of a UVCB (Booth et al., 2023), as was the case for lavender oil in this study, this characterization can be used to reveal biodegradation data for an enlarged number of identified constituents, which can then potentially feed into and improve *in silico* methods. Given that many essential oils are formed and extracted by the same physical/chemical processes, there is an overlap in chemical composition between essential oils, and information on minor constituents may, therefore, be of high value for evaluation of other essential oils (i.e., read across), even in cases where the additionally covered fraction of UVCB mass is small.

Testing multiple chemicals in mixtures and then determining primary degradation for each constituent can be utilized in many ways and potentially be a game changer for biodegradation and persistence assessments. The approach was here applied to two essential oils that served as model UVCBs. In a parallel study, Møller et al. (2024) demonstrated how this approach could be applied to determine the biodegradation kinetics of chemicals that are discharged in produced water from offshore oil platforms to the Sea. Future research should now be directed at combining the new testing approach with the latest developments in non-targeted analysis using GC and LC combined with high-resolution mass spectrometry. Biodegradation kinetics can then be determined simultaneously for hundreds to thousands of chemical features in chromatograms from biodegradation tests of UVCBs or effluents to guide identification of the most persistent constituents or degradation products.

4. Conclusions

This study introduces a whole UVCB testing method with constituent-specific analysis to aid the persistence assessment of UVCBs at low environmentally relevant concentrations. We distinguished biodegradation from abiotic degradation (and loss processes) through the inclusion of both abiotic controls and freshly spiked controls. The careful selection of test mixture concentration combined with sensitive analytical instruments ensured the detection of biodegradation kinetics of an enhanced range of essential oil constituents in a simulation-type biodegradation test. Lastly, the study demonstrated that wastewater

treatment plant-adapted microorganisms and native stream water microorganisms were equally competent degraders for all measured essential oil constituents. The new method can facilitate the substitution of chemicals of concern and the promotion of safe and sustainable-by-design chemicals in the fragrance chemistry market. Ideally, natural origin substances should be as easy to assess as synthetic fragrance chemicals to ensure that substitution decisions are based on a sound scientific basis and not limited by experimental, analytical, and regulatory constraints.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used Grammarly in order to correct grammar. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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CRediT authorship contribution statement

Heidi Birch: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Karen Scharling Dyhr:** Writing – review & editing. **Sylvain Antoniotti:** Writing – review & editing. **Marina Thierry:** Investigation. **Aurelia Lapczynski:** Writing – review & editing. **Philipp Mayer:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

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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Abbreviations

UVCBs	Substances of Unknown or Variable composition, Complex reaction products, and Biological materials
WWTP	Wastewater Treatment Plant
GC-MS	Gas Chromatography-Mass Spectrometry
GC-MS/MS	Gas Chromatography – Triple Quadrupole Mass Spectrometry
OECD	Organization for Economic Co-operation and Development
IPP	Isopentenyl pyrophosphate
DMAPP	Dimethylallyl pyrophosphate
GPP	Geranyl pyrophosphate
DOC	Dissolved Organic Carbon
TSS	Total Suspended Solids
PTFE	Polytetrafluoroethylene
SPME	Solid Phase Microextraction
PDMS	polydimethylsiloxane
MRM	multiple reaction monitoring
FID	flame ionization detector
ECHA	European Chemicals Agency

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2024.143675>.

Data availability

Data has been shared in Supplementary Material.

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