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Article

Passive Dosing of Petroleum and Essential Oil UVCBs—Whole Mixture Toxicity Testing at Controlled Exposure

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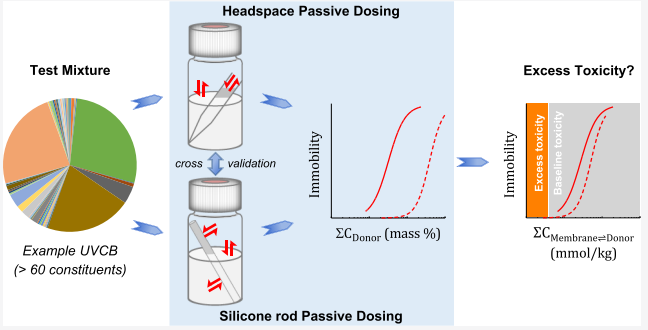
ABSTRACT: Petroleum products and essential oils are produced and used in large amounts and are categorized as "Substances of Unknown or Variable composition, Complex reaction products or Biological materials (UVCBs)." These UVCBs are notorious difficult-to-test substances, since they are complex mixtures of hydrophobic and volatile compounds. This study introduces two passive dosing (PD) approaches for whole UVCB toxicity testing: (1) headspace PD applies the UVCB and purified lipid oil as a donor to control exposure via the headspace and (2) silicone rod PD applies UVCB-loaded silicone rods to control exposure via an aqueous test medium and headspace. Headspace gas chromatography−mass spectrometry measurements were used to cross-validate the approaches at the saturation level and to confirm exposure and maintain mixture composition at varying donor concentration levels. Both approaches were applied to whole-mixture toxicity tests of petroleum and essential oil UVCBs with daphnia and algae. Finally, the observed toxicity was linked to concentrations in the donor and in lipid membranes at equilibrium with the donors. Dose−response curves were similar across the dosing approaches and tested species for petroleum products but differed by an order of magnitude between essential oils and PD systems. All observed toxic effects were consistent with baseline toxicity, and no excess mixture toxicity was observed.



INTRODUCTION

Many chemical substances are in fact complex mixtures that under the European chemical regulatory framework are categorized as "Substances of Unknown or Variable composition, Complex reaction products or Biological materials (UVCBs)."[1] UVCBs have been estimated to comprise about 21% of substances registered at the European Chemicals Agency.[2] Petroleum products and essential oils are two prominent UVCB groups with high annual production volumes. The worldwide production of petroleum products, such as gasoline, kerosene, and diesel, was over 2000 million tonnes in 2016.[3] Essential oils are used in food, beverages, perfumes, pharmaceuticals, and cosmetics.[4] They had an estimated global annual production of more than 150,000 tonnes in 2017, and the production was expected to reach 370,000 tonnes by the 2020s.[4] Emissions to the environment are inevitable. It is thus crucial that appropriate methods are developed for environmental testing and assessment of such UVCBs.[5,6] This is however challenging because they can consist of many constituents, the chemical composition is partly unknown, and/or the composition variability is relatively large or poorly predictable.[1] For example, essential oils can contain from 20 to hundreds of chemical constituents and the compositions are influenced by many factors including climate, plant nutrition, and stress.[7,8] Petroleum products can contain thousands of constituents and the chemical composition varies widely depending on the crude oil origin and operation conditions during refinement.[9,10] Each UVCB constituent has its own set of physicochemical properties (hydrophobicity, volatility, etc.), and UVCBs can contain constituents spanning a wide range in water solubility and partitioning behavior, which in turn sets high demands during UVCB testing and assessment.

One important step in the environmental hazard and risk assessment of chemicals is the aquatic toxicity testing.[11] The first testing challenge is to introduce the UVCBs into the test media in a well-defined and controlled way. Cosolvent spiking is a simple approach to transfer a defined mass and composition of the mixture into the test solution, but it can lead to precipitation of the least soluble components and introduces the solvent into the test medium. Water accommodated fraction (WAF) is another approach that has been developed for preparing aqueous test solutions for petroleum UVCBs.[12] Defined amounts of the UVCB (i.e.,

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loading) are added directly to water and mixed for a period of time sufficient to achieve an equilibrated concentration of dissolved and dispersed or emulsified components in the aqueous phase.[13] A series of WAFs with increasing loadings can then be used for aquatic toxicity testing. Aquatic toxicity testing of WAFs is relevant to petroleum UVCBs since WAFs to some degree resemble oil spill exposures that are governed by dissolution, partitioning, and depletion, leading to varying concentrations and compositions with varying loading levels and times.[14−16] WAFs are the result of several processes, and the resulting aqueous exposure (i.e., concentration and composition) is thus a dependent test variable that is poorly controlled. The second testing challenge is to maintain the initial test exposure throughout the entire test duration. Chemicals with high air−water partition coefficients will partition from the test media into the headspace of the test system, and sorptive and evaporative losses can lead to substantial decreases in concentrations and changes in the mixture composition during the test.[17−19] Declining test concentrations and a changing composition can compromise the interpretation of toxicity results.[13] There is thus a need for dosing methods that facilitate aquatic UVCB toxicity testing at controlled exposure, which requires that (1) the initial exposure is set in a defined and reproducible way, (2) concentration levels can be changed while keeping the mixture composition constant or at least well defined, and (3) exposure can be kept constant during the test duration.

Passive dosing (PD) is increasingly applied to control the aqueous exposure to hydrophobic test chemicals in various toxicity tests.[20−26] The test chemical is loaded to a PD donor, which is then used to establish and maintain constant exposure throughout the test period. The donor capacity is dimensioned to dominate the partitioning of the test chemical within the test system and to avoid donor depletion during the test. The exposure levels can be controlled by varying the concentration in the PD donor (i.e., loading level),[21,27] without changing the composition.[28] PD has mainly been used in research and tests of individual chemicals but has more recently also been applied to defined mixtures with a limited number of constituents.[29−31] An important next step is to develop, optimize, and validate PD approaches for complex mixtures of hydrophobic chemicals[15,32] and then to apply them for improved UVCB exposure control of toxicity tests.[33−35] We are in the present study focusing on the challenge of UVCBs containing volatile constituents that are prone to evaporative losses, which sets new demands for replenishing media and headspace concentrations right after adding the test organisms and also during the test.

In this study, we developed and applied two complementary PD approaches for aquatic toxicity testing of UVCBs that contain hydrophobic and volatile constituents. The first approach is based on phase partitioning of hydrophobic and volatile chemicals via the headspace and is hence termed headspace PD (HS-PD).[26,27] This approach was recently developed for individual chemicals, and it was applied here for the first time to complex mixtures: the neat UVCB was used as the PD donor for controlling defined mixture exposure at saturation, whereas UVCB diluted in a purified lipid oil was used as a PD donor for controlling lower UVCB exposure levels without changing the mixture composition. The second approach applies polydimethylsiloxane (PDMS) silicone rods as the donor and is hence termed silicone rod PD. The silicone rods were directly immersed in an excess amount of UVCB to load them to saturation, and the resulting weight gain (i.e., maximum swelling) was then used as a reference for loading rods for the lower exposure levels. Finally, the loaded silicone rods were placed upright in a test vial for dual-mode PD, meaning direct PD of the aqueous medium and PD via the headspace.

The present study also presents a simple and practical approach to check for excess toxicity of UVCBs relative to baseline toxicity. Baseline toxicity occurs at a critical membrane concentration of 40−160 mmol/kg lipid,[36] and toxicity observations at lower concentrations indicate excess toxicity by other modes of action. We thus relate the observed mixture toxicity to equilibrium concentrations in lipid membranes, which are calculated using mixture-specific correction factors for partitioning differences between the PD donor and the lipid membrane. Toxicity exerted at calculated equilibrium concentrations of 40−160 mmol/kg lipid or higher indicates baseline toxicity, whereas toxicity below this range indicates excess toxicity.

The aims of the present study are: (1) to develop two PD methods for UVCBs that contain volatile hydrophobic chemicals and apply them to mixture toxicity testing, (2) to investigate whether the mixture composition changes with varying concentrations, and (3) to determine the aquatic toxicity of three essential oil UVCBs and three petroleum UVCBs using baseline toxicity as a reference. The respective hypotheses are: (i) both PD methods can control the exposures to the test UVCBs at and below their saturation level, (ii) the mixture composition is largely maintained at varying donor concentrations, and (iii) the tested UVCBs do not exert excess toxicity to *Daphnia magna* (*D. magna*) and *Raphidocelis subcapitata* (*R. subcapitata*).

MATERIALS AND METHODS

Test Mixtures and Materials. Essential oils were provided by Givaudan (Switzerland): fir oil Siberia (CAS 8021-29-2/91697−89-1; batch #AS00331330), cedarwood oil USA Virginia type Orpur (CAS 8000-27-9/85085-41-2; batch #AS00254371), and lavender oil Barreme type (CAS 8000-28-0/84776-65-8; batch #AS00329634). Diesel multiparameter certified reference material CRM-MPGO (CRM-Diesel; CAS 68334-30-5) and jet aviation fuel distillation standard CRMU-DIKR (CRM-Kerosene; CAS 91770-15-9) were purchased from Paragon Scientific Limited (UK). A cracked gas oil (CAS 64741-82-8) was provided by CONCAWE, and it was a complex combination of hydrocarbons obtained from the distillation of products from a thermal cracking process. It consists predominantly of C_{10}−C_{21} unsaturated hydrocarbons in the boiling point range of 160−370 °C. Miglyol oil 812 (Cremer Oleo GmbH, Germany) is a purified plant oil that combines high purity and low viscosity. This purified lipid oil was thus used as a PD donor for HS-PD. Silicone rods (PDMS) with a diameter of 3 mm were custom-made by Altec Product Ltd. (U.K.). This translucent silicone rod does not contain any coloring agent nor filler, which makes it a better defined PD donor and partitioning reference phase compared to the often used red silicone O-rings that contain small amounts of iron oxide (coloring agent) and considerable amounts of diatomaceous earth (filler for physical strength).[37]

Air−water, lipid−water, silicone−water, and membrane−water partition coefficients of representative UVCB constituents were determined using the UFZ database[38] and were applied to calculate additional partition coefficients. Table 1

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Table 1. Partition Coefficients (L/L) of Main Constituents of Essential Oils and Selected Representative Constituents of Kerosene between Various Phases in PD Systems[c]

constituents	CAS No.	log K_{AW}[a]	log K_{LW}[a]	log K_{SW}[a]	log K_{MW}[a]	log K_{LA}[b]	log K_{SA}[b]	log K_{LM}[b]	log K_{SM}[b]	log K_{LS}[b]
fir oil										
camphene	79−92-5	0.92	4.58	4.12	4.06	3.66	3.20	0.52	0.06	0.46
α-pinene	80−56-8	1.23	4.83	4.36	4.24	3.60	3.13	0.59	0.12	0.47
3-carene	13,466−78-9	1.03	4.98	4.44	4.40	3.95	3.41	0.58	0.04	0.54
cedarwood oil										
α-cedrene	469−61-4	1.61	7.25	6.51	6.35	5.64	4.90	0.90	0.16	0.74
thujopsene	470−40-6	0.94	6.76	6.03	5.87	5.82	5.09	0.89	0.16	0.73
cedrol	77−53-2	−2.45	4.71	3.72	4.66	7.16	6.17	0.05	−0.94	0.99
lavender oil										
linalool	78−70-6	−2.45	2.24	1.77	2.40	4.69	4.22	−0.16	−0.63	0.47
linalyl acetate	115−95-7	−0.92	4.11	3.73	3.75	5.03	4.65	0.36	−0.02	0.38
kerosene—hydrocarbon block										
n-P										
n-decane	124−18-5	2.53	6.29	5.79	5.45	3.76	3.26	0.84	0.34	0.50
n-dodecane	112−40-3	2.84	7.43	6.83	6.43	4.59	3.99	1.00	0.40	0.60
i-P										
2-methylnonane	871−83-0	2.60	6.15	5.70	5.34	3.55	3.10	0.81	0.36	0.45
2,2,4,6,6-pentamethylheptane	13,475−82-6	3.19	6.79	6.42	5.89	3.60	3.23	0.90	0.53	0.37
2,2,4,4,6,8,8-heptamethylnonane	4390-04-9	3.92	8.86	8.36	7.67	4.94	4.44	1.19	0.69	0.50
MN										
1,1,3trimethylcyclohexane	3073-66-3	2.06	5.18	4.77	4.54	3.12	2.71	0.64	0.23	0.41
DN										
decalin	91−17-8	1.37	5.68	5.03	5.03	4.31	3.66	0.65	0.00	0.65
cyclohexylcyclohexane	92−51-3	1.06	6.31	5.60	5.66	5.25	4.54	0.65	−0.06	0.71
Mar										
p-xylene	106−42-3	−0.23	3.26	2.83	3.05	3.49	3.06	0.21	−0.22	0.43
3-isopropyltoluene	535−77-3	0.11	4.16	3.69	3.81	4.05	3.58	0.35	−0.12	0.47
1,3,5-triethylbenzene	102−25-0	0.41	5.26	4.70	4.75	4.85	4.29	0.51	−0.05	0.56
NMAr										
1,2,3,4-tetrahydronaphthalene	119−64-2	−0.61	4.14	3.50	3.86	4.75	4.11	0.28	−0.36	0.64

[a]Data from the UFZ-LSER database.[38] [b]Calculated based on corresponding partition coefficients from ([a]). [c]*n-P*, n-paraffin; *i-P*, isoparaffin; *MN*, mononaphthenic; *DN*, dinaphthenic; *MAr*, monoaromatic; *NMAr*, naphthenic monoaromatic; AW, air−water; LW, storage lipid−water; SW, silicone (PDMS)−water; MW, membrane lipid−water; LA, storage lipid−air; SA, silicone−air; LM, storage lipid−membrane lipid; SM, silicone−membrane lipid; and LS, storage lipid−silicone.

lists the governing partition coefficients for the main constituents of the essential oils (>10% in weight, Tables S1−S3) and hydrocarbons from the test substance list of Birch et al.'s study[31] selected here to represent the partitioning behavior of kerosene by covering the hydrocarbon blocks of kerosene (>1%) as reported by Redman et al.[10]

Headspace PD (HS-PD). The aqueous exposure was controlled by partitioning from a liquid donor via the headspace. A pure liquid UVCB served as a donor at the saturation level, whereas dilutions in purified lipid oil (miglyol oil, weight %) were used for dose−response testing. An amount of 0.25 mL of the donor solution was added to a 0.4 mL glass insert containing cleaned glass wool to increase the donor surface area. This insert was then placed upright in a 20 mL clear glass vial before adding the aqueous test media. For the daphnia immobilization test, 10 mL of M7 medium was added and two loaded inserts were used in each test vial, while for the algal growth inhibition test, 4 mL of algae medium and one loaded insert were used. The number of inserts was chosen to ensure sufficient donor capacity and avoid depletion of constituents from the donor. The test vials were closed with airtight PTFE-lined septum screw caps and shaken at 150 rpm (10 mm orbit) in the dark at 20 ± 1 °C for 24 h to pre-equilibrate the test system prior to toxicity tests.

Silicone Rod PD. The silicone rod was first cleaned as described in previous studies[18,26] and then cut into 4.5 cm pieces weighing 0.5 g. The silicone rod was loaded to saturation by immersion in excess UVCB for 48 h. The maximum swelling in mass % is summarized in Table S4. This loading time was set based on reported equilibration times ($t_{95\%}$) of less than 10 h for essential oil and petroleum UVCBs[15] and confirmed by measurements after 24 and 48 h of loading (Table S4). Loadings below saturation were obtained by full absorption of smaller amounts of UVCB. These loadings were done in 20 mL autosampler vials at 20 ± 1 °C using a tube roller (Ratek Instruments, Australia) for at least 48 h. The loaded rods were wiped with a lint-free tissue (Assistant, Germany) and weighed again to determine the exact mass % of UVCBs loaded. They were then placed upright in the 20 mL test vials, which resulted in PD by mass transfer via the headspace and directly via the silicone−water interphase. Two loaded rods and 10 mL of M7 medium were added to each test vial for the daphnia test, and one loaded rod and 4 mL of algae medium were added for the algal test. Pre-equilibration similar to that described for HS-PD was carried out before starting the toxicity tests.

Mass Balance Calculations. Mass distribution of representative constituents within the test system was

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determined based on mass balance calculations, with and without a PD donor. These calculations confirmed that the PD donor was necessary and sufficient to control the partitioning of the constituents in the closed test system (Table S5). The mass balance calculations were conducted using a 20 mL test vial, 10 mL of water, and either 0.5 mL of lipid or 1 mL of silicone donor. The applied partition coefficients are listed in Table 1.

Acute *D. magna* Immobilization Test. *D. magna* culture was obtained from DHI (Hørsholm, Denmark). About 12−15 daphnids were maintained in 1 L glass beakers containing 800 mL of M7 medium, which was prepared according to the OECD Guideline 202.[39] The culture was fed with the green algae *R. subcapitata* and maintained at 20 ± 1 °C with a 12:12 h light/dark regime. Neonates were separated every second day, and the medium was renewed twice a week.

The 48 h acute *D. magna* immobilization test was based on the OECD Guideline 202.[39] The test neonates were less than 24 h old, and only from the third to sixth brood. Both PD methods were applied to control the exposures to three essential oils and three petroleum UVCBs. For headspace PD, donor solutions were prepared with 1, 3, 10, and 30% (mass:mass) of the UVCBs in miglyol oil and with 100% UVCB. In the silicone rod PD, the UVCBs were loaded to silicone rods to 1, 3, 10, 30, and 100% of the maximum loading. All tests were carried out with four replicates with five neonates each for all the treatments including the global control (no insert or rod), the oil control (inserts with miglyol oil), and the silicone control (clean silicone rods). At the starting of the test, the pre-equilibrated test vials were opened shortly one by one for adding the neonates, and the test vials were then kept in the dark at 20 ± 1 °C. Daphnids were considered immobilized if they were not able to actively swim within 15 s after gently shaking the test vial. Finally, the sigmoidal Hill function with a variable slope was fitted to the immobility data using the least-squares method (GraphPad Prism 5.0).

***R. subcapitata* Algal Growth Inhibition Test.** Both PD approaches were applied to control the exposures to fir oil and CRM-Diesel in closed algal growth inhibition tests with CO_2-enriched headspace.[26,40] The same concentration ranges were applied as for the daphnia immobilization test. Each test consisted of six global control replicates (no insert or silicone rod) and three replicates for the other treatments including the oil control (insert with miglyol oil) and the silicone control (clean silicone rod). The mean growth rates of the oil control and silicone control samples were used as a reference for calculating the inhibition in the HS-PD and silicone rod PD methods, respectively. The growth inhibition was calculated as described earlier.[26] Finally, the sigmoidal Hill function with a variable slope was fitted to the growth inhibition data by the least-squares method (GraphPad Prism 5.0).

Calculation of the Mixture Concentration in Membranes at Equilibrium with the Lipid and Silicone Donors. The mass concentrations in the lipid and silicone donors ($C_{\%,donor}$) were the controlled and best defined variables in the toxicity tests. $C_{\%,donor}$ values were converted to mmol/kg donor (C_{donor}) using the average molecular weight (g/mol) of the test mixture.[27] Based on the available compositions of the essential oils (Tables S1−S3), the average molecular weight was calculated to be 138, 157, and 188 g/mol for fir oil, lavender oil, and cedarwood oil, respectively. For the petroleum UVCBs, all conversions were made with an assumed average molar weight of 170 g/mol because of the large number of constituents and the limited compositional information.

The molar concentrations in the donor were then converted to equilibrium membrane concentrations in order to correct for the moderate partitioning differences that exist between silicone, miglyol, and lipid membranes. For individual constituents, these calculations were carried out using the partition coefficients listed in Table 1. For the whole UVCBs, concentrations in the membranes at equilibrium with the lipid donor were approximated using the mean Log K_{LM} values of the representative constituents (Table 1). Concentrations in membranes at equilibrium with the silicone donor were approximated using the mean Log K_{SM} values of the representative constituents (Table 1). The mean Log K_{LM} and Log K_{SM} values of kerosene were applied to the two other petroleum UVCBs. These calculations did not account for density differences between the donor phase and lipid membranes nor that slightly higher Log K_{LM} and Log K_{SM} are expected for the larger molecular constituents of diesel and cracked gas oil. Overall, the calculated concentrations in the lipid and the membrane are associated with significant numerical uncertainties and should thus be assessed and applied on a logarithmic rather than a linear scale.

Exposure Cross-Validation at Saturation. The UVCB exposure generated by HS-PD and silicone rod PD was determined at the saturation level using separate test vials (without test organisms) to cross-validate these two methods. The test mixtures were measured in the headspace of both systems and then compared to their saturated vapors over the pure liquid UVCB, which served as the analytical and thermodynamic reference.[27] The two PD systems were prepared as described earlier but with 20 mL autosampler vials instead of 20 mL toxicity test vials and with 8 mL of M7 medium. The reference systems were prepared by adding 2 mL of UVCBs to an autosampler vial. A gastight syringe operated by the GC autosampler was then used to inject 100 μL of headspace air to the gas chromatograph−mass spectrometer (GC−MS).

Mixture Composition at Donor Loadings below Saturation. The exposure level of several fir oil constituents and the resulting mixture composition were determined based on headspace GC−MS analysis at varying loading levels. Constituent-specific exposure levels in the PD systems were determined based on the peak area relative to the peak area in the reference system, which yielded the chemical activity in the PD donor relative to the chemical activity in the UVCB product ($a_j/a_{j,mixture}$).[27] Major changes in the mixture composition with increasing loadings would then become apparent as deviations between the loading−activity curves. This experiment was carried out in three replicates using the HS-PD method. A series of mass concentrations of 2, 5, 10, 25, 50, 80, and 100% of fir oil in miglyol was prepared. Then, 2 mL of each concentration was added to an autosampler vial and headspace GC−MS was employed for analysis.

The relative chemical activity of the selected constituents of fir oil as a function of the mass concentration of fir oil in miglyol (X, %) was fitted using a one-phase association exponential model (eq 1) using least-squares regression (GraphPad Prism 5.0).

$$a_j/a_{j,mixture} = (1 - e^{-kX}) \quad (1)$$

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PD Kinetics. PD kinetics of fir oil and CRM-Diesel were determined for both PD systems to demonstrate pre-equilibration within 24 h and an efficient buffering of test substance concentrations. The donors were prepared at a mass concentration of 20% for the HS-PD (C_{Lipid} = 20%) and 10% for the silicone rod PD ($C_{Silicone}$ = 10%). Two loaded inserts and two loaded rods were used. M7 medium (10 mL) was then added and the test systems were shaken at 150 rpm and 20 ± 1 °C. After 1, 3, 6, 24, and 72 h, 0.5 mL of samples was taken using a 1 mL gastight glass syringe and transferred to 9.5 mL of saline solution (200 NaCl g/L) contained in a 20 mL autosampler vial. The 24 h sampling interval corresponds to pre-equilibration of the PD systems in toxicity tests, while the 72 h sampling interval corresponds to the end of daphnia tests (48 h after the test started). At the 24 h time point, an additional sample was taken after opening shortly and closing the test vial, simulating the addition of daphnids to the test. The sample vials were instantly closed and kept at 4 °C until analysis by headspace solid-phase microextraction (SPME)-GC−MS. This experiment was carried out in triplicate.

Headspace GC−MS Analysis. Headspace GC−MS analysis was used for the cross-validation and mixture composition experiments. Analyses were performed using an Agilent Technologies GC−MSD system (7890B/5877A GC/MSD) with a CTC PAL RSI 85 autosampler (CTC Analytics, Zwingen, Switzerland). The samples were equilibrated at 35 °C at intermittent orbital shaking for 30 min, and 100 μL was then withdrawn from the headspace and injected into the GC−MS. The analyses of the test mixtures at the saturation level were run using splitless injection. The subsequent analyses of fir oil at and below the saturation level were carried out with a split ratio of 10:1 (Figure S2A). The separation was performed using a DB-5 ms Ultra Inert column (60 m × 250 μm × 0.25 μm) that was first maintained at 60 °C for 5 min followed by 1.5 °C/min to 120 °C, 5 °C/min to 220 °C, and 25 °C/min to 320 °C. The MS was operated in the scan mode (50−500 m/z, gain factor of 3). Peak areas of fir oil constituents were manually integrated (MSD ChemStation, Agilent Technologies). The linearity of the response was demonstrated for the full concentration range by additional measurements in the splitless mode for the concentrations 2, 5, and 10% (see Figure S2B).

Headspace SPME GC−MS Analysis. The water samples from the PD kinetic experiment were measured by headspace SPME with a 7 μm PDMS fiber at 35 °C and 250 rpm for 60 min followed by thermal desorption for 10 min at 250 °C. Thermal desorption was performed in the splitless mode for CRM-Diesel, and a split ratio of 20:1 was used for fir oil. The GC oven was held at 40 °C during thermal desorption and temperature was increased by 10 °C/min to 100 °C, 1.5 °C/min to 200 °C, and finally 20 °C/min to 320 °C. The MS was operated in the scan mode (50−500 m/z, gain factor of 3). A reference standard (1% UVCB in miglyol oil) was analyzed for each PD system to account for differences in the instrument sensitivity and retention time drifts between runs. The data treatment was performed using MassHunter Quantitative Analysis version B.09.00/Build 9.0.647.0 for GC−MS and LC−MS. Compounds were tentatively identified by MassHunter Unknowns Analysis of the full scan chromatograms using deconvolution and spectral library search via NIST 17.1. Peaks that were present in blank samples were excluded from further analysis, and the remaining constituents were selected to represent the UVCB within the GC retention time range. Peak heights were obtained by MassHunter Qualitative Analysis and then used to determine the dosing kinetics. The kinetic curves were recorded using GraphPad Prism 5.0 with the one-phase exponential association model.

RESULTS AND DISCUSSION

Cross-Validation of Exposure at Saturation. The initial assessment and confirmation of exposure at saturation were performed by visual inspection of the overlaid total ion chromatograms (TICs) of the two PD systems and the saturated vapor reference (Figure S1). Six main constituents were identified in fir oil based on their distinct peak heights (Figure S1A). Figure 1A shows that their peak areas measured in the two PD systems were similar to their peak areas measured in the saturated vapor reference system and thus on the 1:1 line. This means that the two PD approaches provided the same maximum exposure (i.e., cross-validation), which also agreed with the liquid UVCB that served as an additional reference. The same result was observed visually for cedarwood and lavender oils, of which the overlaid chromatograms were almost identical between the PD systems and the pure products (Figure S1B,C). For petroleum UVCBs, the overlaid chromatograms showed somewhat lower responses for the very first constituents in the chromatograms generated by the silicone PD method compared to the HS-PD method and the pure products (Figure S1D−F). These constituents, however, are very volatile, as they appeared very early in the chromatogram, and thus may have been lost to a minor extent during the physical handling of the loaded silicone rods (e.g., weighing).

Mixture Composition at Varying Donor Loadings. Figure 1B shows the chemical activities of fir oil constituents in lipid donors relative to their chemical activity in the fir oil. As seen previously for single test chemicals,[27] the relationship between the lipid donor loading and the chemical activity was linear up to about 20% loading and then nonlinear at higher loadings. The fitted curves were similar between the main constituents of fir oil (Figure 1). This means a constant mixture composition within the low loading range and a largely maintained mixture composition at higher donor loadings. PD can thus provide well-defined exposures to a multicomponent mixture, such as fir oil, in which the mixture composition is reasonably maintained across exposure levels.

PD Kinetics. The PD kinetics in the aqueous medium were fast for most fir and diesel oil constituents, with equilibration times within 1 h when using silicone rod PD (Figures S3,S5) and 3 h when using HS-PD (Figures S4,S6). The exposure concentrations of these constituents were kept rather constant during the 72 h period. Opening the test vials for test organism addition resulted only in a minor concentration fluctuation compared to the unopened vials (within ±20%). The HS-PD kinetics were markedly slower compared to the silicone rod PD for two fir oil constituents with high GC retention times (35.15 and 35.16 min). This makes good sense since HS-PD is limited to mass transfer via the headspace, whereas silicone rod PD is based on mass transfer via headspace and via the silicone−water interphase. These analytical results confirmed that 24 h pre-equilibration was sufficient for all but two constituents in the HS-PD, the short opening of the test vials did not affect the overall exposure, and constant exposure of test UVCBs was maintained in abiotic test systems throughout the test duration by the HS-PD (with two exceptions) and silicone rod PD.

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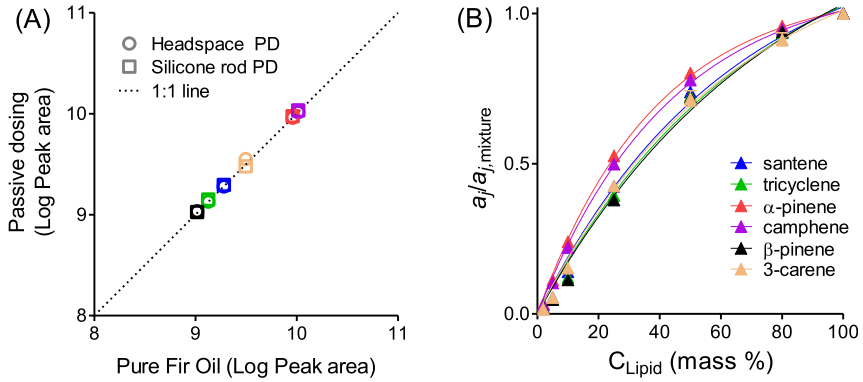

Figure 1. Exposure confirmation. (A) Peak areas of the seven main constituents of fir oil measured in the two PD systems against the same constituents in saturated vapors over the pure fir oil (reference system). (B) Chemical activity (a_j) of main constituents of fir oil relative to their maximum chemical activity in pure fir oil ($a_{j,mixture}$) measured in the gaseous phase of the headspace PD system at different concentrations in the lipid donor (C_{Lipid}).

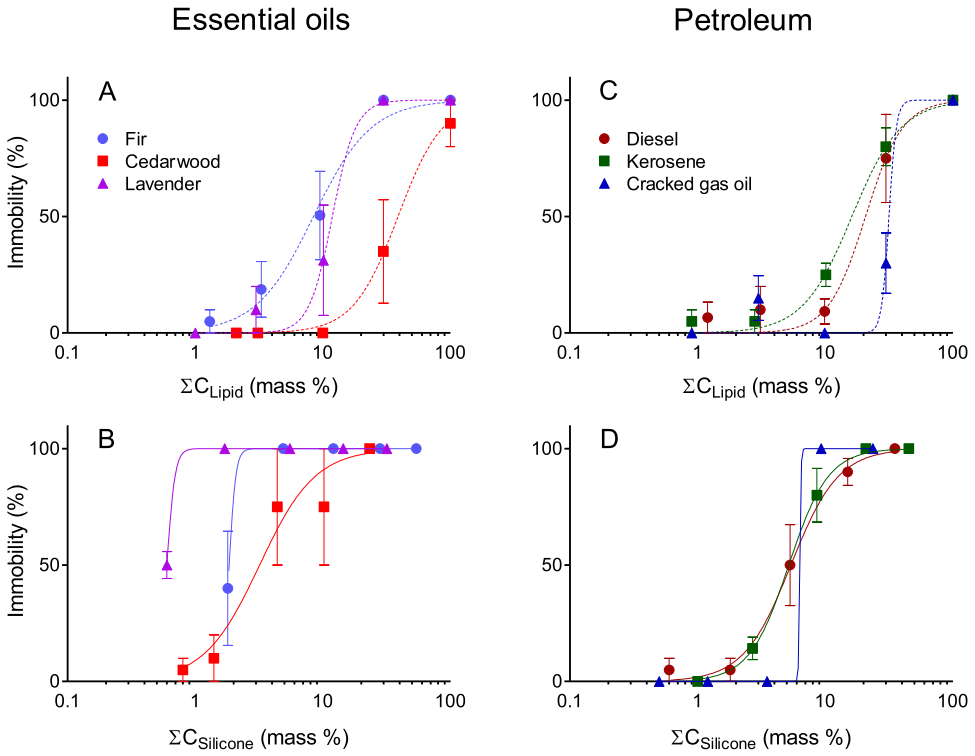

Figure 2. Toxicity of essential oils (A, B) and petroleum products (C, D) to *D. magna* as a function of their mass fraction in the lipid and silicone donors.

Toxicity to *D. magna*. No immobilized daphnids were observed in the three control treatments in any of the acute *D. magna* immobilization tests, (i.e., validity criteria). All test mixtures resulted in 100% immobility at the maximum exposure level for both PD methods (Figure 2). Cedarwood oil was the least toxic among the essential oils in both PD methods (Figure 2A,B), whereas toxicity toward daphnia was similar among the tested petroleum products (Figure 2C,D).

The mixture concentrations in membranes at equilibrium with the PD donor were calculated, and toxicity as a function of these membrane concentrations is shown in Figure 3. All tested UVCBs caused immobilization of daphnids at calculated equilibrium membrane concentrations within or above the reported range for baseline toxicity (40−160 mmol/kg). The daphnia toxicity tests yielded consistent toxicity observations for petroleum products using both PD methods (Figure 3B). For fir oil, the observed toxicity was also similar between PD methods, while for cedarwood and lavender oils, the toxicity was higher when dosing from silicone rods than through headspace (Figure 3A). However, the toxicity of cedarwood and lavender oils obtained with the silicone rod PD method was still within the baseline toxicity range. These results hence support the hypothesis that the tested UVCBs did not exert excess toxicity toward *D. magna*. It cannot be ruled out that some of the observed dose−response curves were somewhat right shifted because of internal concentrations in the daphnids not reaching equilibrium within the test duration. Furthermore, biotransformation of some constituents could also have led to internal exposures below the equilibrium level. However, assessing the toxicity of the tested UVCBs based on equilibrium partitioning and baseline toxicity seems still

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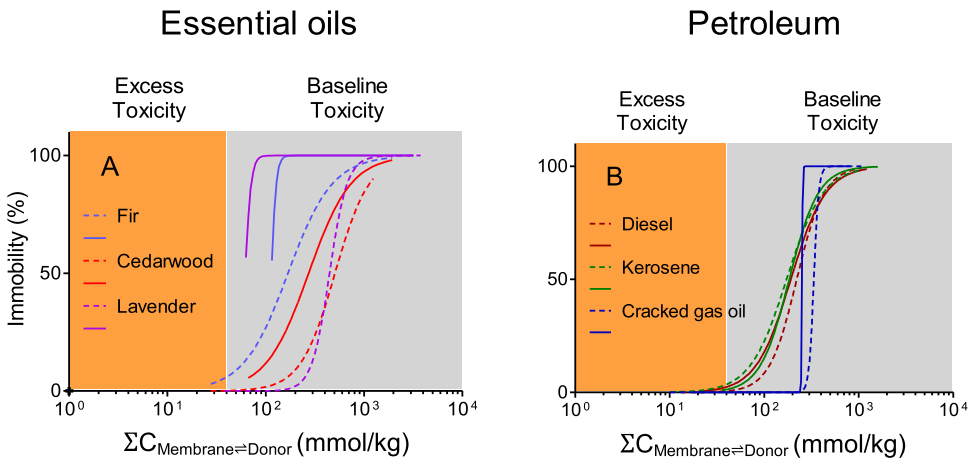

Figure 3. Toxicity of essential oils (A) and petroleum products (B) toward *D. magna* linked to calculated concentrations in membranes at equilibrium with the lipid (dashed line) and silicone (continuous line) donors, $C_{Membrane⇌Donor}$.

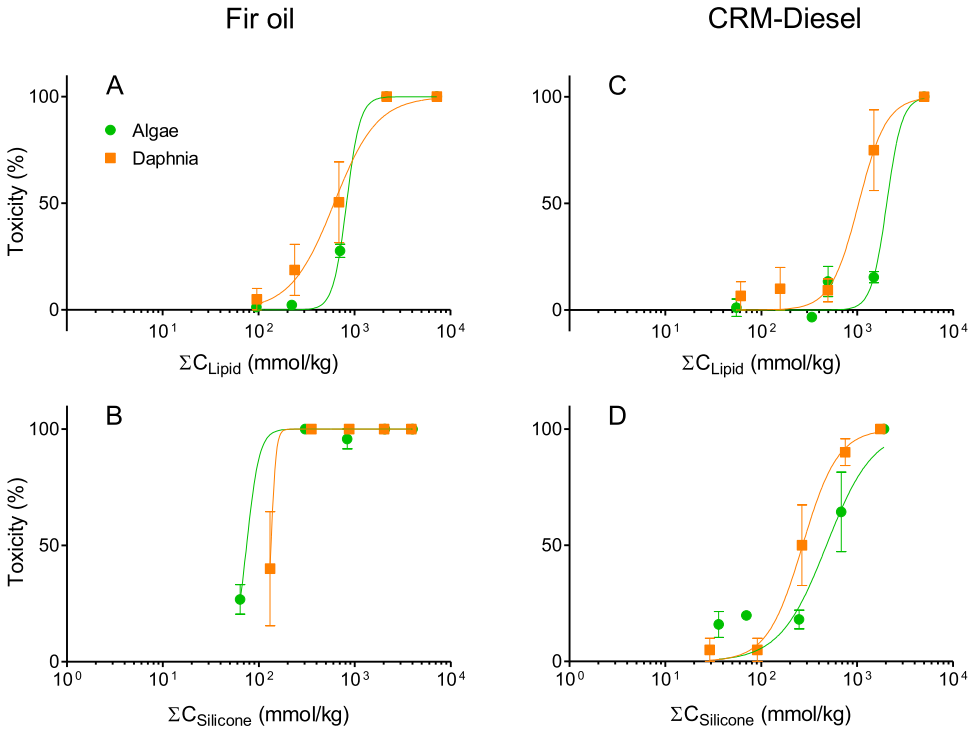

Figure 4. Toxicity of fir oil and CRM-Diesel to *R. subcapitata* and *D. magna* as a function of concentration in lipid (A, C) and silicone (B, D) donors.

adequate or somewhat conservative based on the position of the concentration response curves shown in Figure 3.

The consistency in the toxicity of petroleum products between the HS-PD and silicone rod PD (Figure 3B) cross-validated the two methods on the effect level. For the essential oils, the dose−response curves were somewhat right shifted for the HS-PD compared to the silicone rod PD, which can be explained by slower PD kinetics of the HS-PD approach for some more polar and less volatile constituents. As a result, the dose−response curves for the essential oils based on HS-PD are less valid, and they were mainly included here for illustration and comparison. This highlights the larger applicability domain of the silicone rod PD method for UVCBs containing less volatile constituents.

Toxicity to *R. subcapitata*. The mean growth rates in the oil and silicone controls did not differ statistically from that of the global control in any algal growth inhibition test (Figure S7) and were greater than the validity criterion of 1.4 per day.

Figure 4A,B shows the similar toxicity of fir oil toward algae and daphnia when tested with HS-PD and silicone rod PD methods. This is in contrast to the CRM-Diesel that was more toxic to daphnia than algae (Figure 4C,D) as demonstrated by the statistically lower EC-50 values ($p < 0.05$, Table S6). The toxicity results from the algal growth inhibition tests were again consistent with baseline toxicity. It is important to note that the distinction between baseline and excess toxicity is specific for and thus limited to the specific test species, test duration, and toxicity endpoint.

PD for Toxicity Testing of UVCBs. Both PD methods enabled the whole-substance toxicity testing of UVCBs at controlled exposures. The two methods have different advantages and limitations and can thus complement each

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other. The silicone rod PD method has a much larger applicability domain in terms of chemical space, which will make it particularly applicable to many UVCBs. The less volatile constituents will be most effectively dosed via the silicone−water interphase, whereas the most volatile constituents will be most effectively dosed via the headspace. The observed toxicity can also be related to the sum concentration of the mixture in the silicone donor, which can provide silicone-based effect concentrations that can be directly linked to passive sampling results.[41] The HS-PD technique allows experiments to be set up faster and with less preparative work since the lipid donor is simply loaded by the addition of the UVCB. This technique is well suited for mixtures of volatile constituents that partition well via the headspace, while it can lead to the underestimation of toxicity for UVCBs with a significant nonvolatile fraction.

The presented PD approach was shown to improve the exposure control of UVCBs in aquatic toxicity tests since it allows varying concentrations at a constant composition and buffers the exposure against losses during the test. The observed toxicity was in the present study related to the loaded UVCB concentration in the PD donor and also to calculated equilibrium concentrations in the target membranes. The obtained results were consistent with the hypothesis that the tested UVCBs acted by baseline toxicity. An important next step is to find a practical and sound way to relate the observed toxicity to the controlled aqueous UVCB exposure in the test. We foresee two complementary approaches: (1) Aqueous UVCB concentrations can be measured at the start and the end of the test in order to confirm stable concentrations, while at the same time serving as a basis to determine the EC-50 values. However, such measurements require a substantial analytical effort at least for UVCBs with a high number of constituents. (2) Alternatively, constituent concentrations in the donor can be calculated based on the UVCB composition and the loading level, which can then be converted to aqueous concentrations using constituent-specific donor-to-water partition coefficients. The measured or calculated exposure concentrations can then be used for assessing mixture toxicity and for concentration response fitting. Another important prospect is the chronic toxicity testing of UVCBs, which is more environmentally relevant than acute testing and requires constant exposure at very low concentrations for longer test durations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c00343.

Analysis of exposures at the saturation level for all test UVCBs, kinetics of fir oil and diesel in an aqueous medium, mass balance calculations, linearity check of the GC−MS for the chemical activity analysis, algal growth in the control samples, composition of the tested essential oils, maximum swelling of test mixtures in silicone rods, and EC-50 values of test mixtures in daphnia and algae (PDF)

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Notes

The authors declare no competing financial interest.

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