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HEADSPACE PASSIVE DOSING OF VOLATILE HYDROPHOBIC ORGANIC CHEMICALS FROM A LIPID DONOR – LINKING THEIR TOXICITY TO WELL-DEFINED EXPOSURE FOR AN IMPROVED RISK ASSESSMENT

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2   **LINKING THEIR TOXICITY TO WELL-DEFINED EXPOSURE FOR AN IMPROVED RISK ASSESSMENT**

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12   **Notes**

13   The authors declare no competing financial interest.

14 **ABSTRACT**

15 High hydrophobicity and volatility of chemicals often lead to substantial experimental challenges, but was
16 here utilized in headspace passive dosing (HS-PD) to establish and maintain exposure: The pure chemical
17 served as passive dosing donor for controlling exposure at saturation, whereas triglyceride oil containing
18 the chemical was used to control lower exposure levels. These donor solutions were added to glass inserts
19 placed in the closed test systems. Mass balance calculations confirmed a dominant donor capacity for all
20 chemicals except isooctane. This HS-PD method was applied to algal growth inhibition and springtail
21 lethality tests with terpenes, alkanes, and cyclic siloxanes. Headspace concentrations above the lipid donors
22 were measured for three chemicals to determine their chemical activity, using saturated vapor as analytical
23 standard and thermodynamic reference. Toxicity was related to chemical activity and calculated
24 concentrations in membranes at equilibrium with the lipid donor. For both tests and all chemicals, toxic
25 effects were observed within or above the reported range for baseline toxicity, meaning that no excess
26 toxicity was observed. The toxicity of siloxanes was markedly higher to the terrestrial springtail than the
27 aquatic algae, which is consistent with a more efficient mass transfer of these volatile hydrophobic
28 chemicals in air compared to water.

29 **Keywords:** *Raphidocelis subcapitata*, *Folsomia candida*, aliphatic hydrocarbons, narcosis, Henry's law,
30 Raoult's law.

31 **TOC/ABSTRACT ART**

33 1. INTRODUCTION

34 High volatility and high hydrophobicity make chemicals “difficult to test” with regards to toxicity testing¹.
35 When a chemical possesses both properties, it becomes almost untestable with current standard toxicity
36 test methods. In fact, for volatile hydrophobic chemicals with an air-water partition coefficient (K_{AW})
37 greater than 1 (L/L), a considerable mass fraction will partition into the headspace of aquatic test
38 systems^{2,3}. The exposure via soil interstitial air will become important for terrestrial organisms, because of a
39 more efficient mass transfer via air compared to water^{4–6}. For both aquatic and terrestrial testing, it is
40 generally challenging to first establish well-defined exposure at the beginning of the test, then to maintain
41 constant exposure during the test, and finally to analytically confirm this exposure¹. Experimental strategies
42 to cope with these challenges include (1) flow-through systems with a continuous supply of spiked
43 medium¹, (2) non-depletive testing focusing at minimizing test substance losses², and (3) passive dosing
44 where the concentration in the test is established and re-supplied by phase partitioning from a pre-loaded
45 polymer⁷. Recently, a new headspace passive dosing (HS-PD) approach was developed for enabling aquatic
46 toxicity testing of volatile hydrophobic organic chemicals (VHOCs) at the maximum exposure level⁸. The
47 toxicity tests using HS-PD were conducted in gas tight vials, and the pure liquid test substance was used as
48 a partitioning donor to establish and control the test exposure exactly at the saturation level⁸. The present
49 study was directed at developing and applying a HS-PD approach for the dose-response testing of VHOCs.

50 The exposure to a test substance can be expressed in several ways that go beyond the concentration in the
51 test medium (e.g., air and water). Various approaches have been proposed to link adverse biological
52 responses to the internal exposure, such as the critical body residue model⁹, critical body burdens¹⁰, toxic
53 cell concentrations¹¹, and the target lipid model¹². These studies advocate the use of the critical internal
54 dose that causes 50% mortality in test organisms, e.g., critical body residue CBR-50, for the interpretation
55 and prediction of toxicity. This is due to the fact that external medium-based effective concentrations vary
56 greatly for narcotic chemicals, whereas their CBRs remain within a much more narrow range^{9,13}. It has been

57 reported that nonpolar narcosis initiates at a target concentration range of 40-160 mmol/kg membrane
58 lipid¹⁰, which in the present study is used as a reference for identifying excess toxicity.

59 Another way to interpret and study the toxicity of narcotic chemicals is to link toxic effects to chemical
60 activity^{14,15}. Chemical activity expresses the chemical potential relative to a chosen reference state^{16–19},
61 which for hydrophobic chemicals often is the pure chemical in its liquid state where chemical activity is set
62 to unity¹⁶. The chemical activity is then defined between 0 and 1, and differences in chemical activities
63 between different phases drive the diffusion, sorption, and partitioning of the chemical, with a direction
64 from high to low chemical activity¹⁶. Therefore, chemical activity can give new insights and facilitate
65 comparison of chemical exposures across environmental compartments including air, water, soil, sediment,
66 and biota. The relationship between chemical activity and toxicity was first proposed by Ferguson in the
67 late 1930's¹⁴, and several studies have since shown that nonpolar narcotic chemicals exert baseline toxicity
68 when reaching a chemical activity between 0.01-0.1^{15,20–22}. Specific toxicity or excess toxicity is thus
69 indicated when effects occur at a lower chemical activity (< 0.001)^{19,23}.

70 The link between the toxicity of hydrophobic chemicals and internal exposure or chemical activity has been
71 explored more recently with the use of passive dosing, which allows precise exposure control^{7,13,20,24–30}.
72 Since passive dosing is based on equilibrium partitioning of the test chemical between different phases, its
73 application in toxicity tests has several advantages: (1) by controlling concentrations in the donor, the
74 concentrations in the test medium are precisely controlled, (2) the theoretical equilibrium partitioning
75 concentration of test chemical in the organisms can be estimated, and (3) the chemical activity in the test
76 system is determined by the concentration in the donor and can be measured in the headspace.

77 The present study introduces the use of a lipid as passive dosing donor for the toxicity testing of liquid
78 VHOCs at and below their saturation level. Lipid has recently been successfully applied as a partitioning
79 donor in dedicated analytical and partitioning studies with semivolatile organic chemicals^{31–33}, while other
80 scientists used vacuum pump oil as partitioning donor for controlling headspace concentrations in

autosampler vials for calibration purposes^{34,35}. The working principle of the new passive dosing method is based on equilibrium partitioning between donor and exposure medium, without a direct contact between these two phases. The test system is simple: The lipid donor is loaded by direct addition of the liquid test chemical and then used for establishing and controlling exposures below the saturation level, while the test chemical in the pure liquid form is used to establish and control exposure at the saturation level. The donor in this passive dosing approach is kept separate from the test medium and organisms using a glass insert (see the TOC Figure). We used purified plant oil as lipid donor, which is a medium-chain triglyceride of fractionated plant fatty acids, e.g., caprylic (C8) and capric (C10) fatty acids. There are a number of advantages when using such a liquid lipid as donor: (1) it is easy to load these high capacity donors to different chemical concentrations for toxicity testing by directly mixing the test chemical with the purified plant oil, (2) it allows measurements of chemical activity based on headspace concentrations of test chemicals, (3) it allows prediction of “concentrations in membranes at equilibrium with the donor” based on estimated storage lipid to membrane lipid partition coefficients, and (4) toxicity can finally be linked to both chemical activity and calculated equilibrium concentrations in membranes.

The aims of the present study were: (1) to develop a passive dosing method that can control the exposure to VHOCs at and below their saturation level, (2) to apply the method to dose-response testing of mono terpenes, alkanes, and cyclic volatile methyl siloxanes with the freshwater green algae *Raphidocelis subcapitata* (Sphaeropleales; Selenastraceae) and the terrestrial springtail *Folsomia candida* (Collembola; Isotomidae), and (3) to link the toxic effects to calculated equilibrium concentrations in membranes and to the measured chemical activities, and thus assess whether the test chemicals exert baseline or excess toxicity. The hypotheses were that: (i) the HS-PD approach can control exposure concentrations at and below the saturation level in algal growth inhibition test and springtail lethality test, (ii) the tested chemicals do not exert excess toxicity, and (iii) since chemicals with high K_{OW} and high K_{AW} have more efficient mass transfer in air compared to water, such chemicals can have faster uptake kinetics in the absence of water and thus higher toxicity in small terrestrial compared to aquatic organisms.

2. MATERIALS AND METHODS

2.1. Chemicals and materials

The following test chemicals were from Sigma-Aldrich Denmark with purity $\geq 99\%$ unless otherwise stated:

α -(+)-pinene, α -(-)-pinene, S-(-)-limonene (96%), octamethylcyclotetrasiloxane (D4) (98%), decamethylcyclopentasiloxane (D5) (97%), isooctane, n-nonane, n-undecane (Sigma-Aldrich, Germany), n-dodecane and isododecane (98%) (TCI Chemicals, Belgium), and n-tridecane (Fluka AG, Switzerland). Miglyol 812 purified plant oil was obtained from Cremer Oleo GmbH, Germany.

2.2. Headspace passive dosing using lipid as donor

2.2.1. Headspace passive dosing system

Test chemicals were weighed and mixed into Miglyol oil (without any pre-treatment of the oil) to obtain desired test concentrations (% w/w, mass %), while pure liquid test chemicals with no further treatment were used to control exposure at the saturation level according to Trac and colleagues⁸. An amount of 0.2 mL of the donor solutions was added to 0.3-mL glass inserts embedded with glass wool to increase the surface area for the passive dosing. The inserts were then placed in 20-mL test vials containing 4 mL algal test medium or in 20-mL empty test vials for the springtail lethality experiment (further details in Section 2.3 and depicted in the TOC Figure). The test vials were then closed air tight with screw caps with Teflon-lined (PTFE) septa, and finally the complete systems were kept at 20 ± 1 °C for 24 h for pre-equilibration. During this period, the closed test vials were shaken at 200 rpm (10 mm orbit) in order for the test chemicals to equilibrate quickly within the test system. Shaking of test vials was not necessary for springtail lethality tests. At test start, the pre-equilibrated vials were opened shortly for adding test organisms. The loss of test chemical during the opening was assumed to be limited to the fraction in the headspace, which was negligible as demonstrated by the mass balance calculations below.

2.2.2. Mass balance calculations

129 Mass balance calculations were done for an algal toxicity test system with and without passive dosing
130 donor (Table 1) in order (1) to illustrate the differences between the two systems and (2) to demonstrate
131 that the donor capacity was sufficient for controlling the partitioning within the closed system (i.e.,
132 negligible donor depletion). The calculations were done for all test chemicals using partition coefficients
133 from the UFZ-LSER database³⁶, a donor volume of 200 µL, a water volume of 4 mL, and a vial volume of 20
134 mL (see Section 2.2.1).

135 **Table 1:** Partition coefficients of the test chemicals and their mass balances calculated for test systems with
136 and without a passive dosing donor. PD: passive dosing, OW: octanol-water, AW: air-water, LW: storage
137 lipid-water, MW: membrane lipid-water, LA: storage lipid-air.

Compound	Governing partition coefficient					Mass balance without PD ^c		Mass balance with PD ^c		
	Log <i>K</i> _{OW} ^a	Log <i>K</i> _{AW} ^a	Log <i>K</i> _{LW} ^a	Log <i>K</i> _{MW} ^a	Log <i>K</i> _{LA} ^b	Air	Water	Lipid	Air	Water
n-nonane	5.46	2.38	5.72	4.96	3.34	99.9%	0.10%	96.5%	3.49%	< 0.01%
n-undecane	6.57	2.69	6.86	5.94	4.17	99.9%	0.05%	99.5%	0.53%	< 0.01%
n-dodecane	7.13	2.84	7.43	6.43	4.59	100.0%	0.04%	99.8%	0.20%	< 0.01%
n-tridecane	7.68	3.01	7.98	6.90	4.97	100.0%	0.02%	99.9%	0.08%	< 0.01%
isooctane	4.65	2.40	4.81	4.19	2.41	99.9%	0.10%	76.5%	23.50%	0.02%
isododecane	6.65	3.19	6.79	5.89	3.60	100.0%	0.02%	98.1%	1.95%	< 0.01%
Limonene	4.44	0.54	4.60	4.09	4.06	93.3%	6.73%	99.3%	0.68%	0.05%
α-pinene	4.61	1.23	4.83	4.24	3.60	98.5%	1.45%	98.0%	1.95%	0.03%
D4	6.92	3.21	6.61	5.65	3.40	100.0%	0.02%	97.0%	3.05%	< 0.01%
D5	8.07	3.52	7.51	6.39	3.99	100.0%	0.01%	99.2%	0.80%	< 0.01%

138 ^a data from UFZ-LSER database³⁶

139 ^b calculated by subtracting Log *K*_{LW} by Log *K*_{AW}

140 ^c due to rounding numbers may add up to more or less than 100%

141 The mass balance calculations in Table 1 apply to the Henry’s law regime with donor concentrations up to
142 30%, which complements the mass balance calculations for the saturation level in Trac et al.⁸. In the air-
143 water system, all chemicals predominantly partition into the headspace, which asks for and justifies

exposure confirmation to be done by headspace analysis. In the passive dosing system, 200 μL of storage lipid was found to have sufficient donor capacity for the passive dosing of water and headspace with donor depletion $< 10\%$ for all chemicals except isooctane. The high capacity of the Miglyol oil is consistent with lipid-water partition coefficients being considerably higher than K_{OW} and K_{MW} . The donor depletion in the Henry regime (Table 1) is higher than in the saturation regime, and can thus be used as a conservative estimate.

2.2.3. Analytical exposure confirmation

This experiment was performed to (1) analytically determine the chemical activity of three test chemicals in the HS-PD system relative to their saturation level, and (2) link the test chemical concentrations in the lipid donor to chemical activity and thus determine the actual chemical activity of these test chemicals in the toxicity tests. This exposure analysis was performed for limonene, n-nonane, and D4, chosen as representatives for the chemical groups tested. A series of concentrations in Miglyol oil (mass %) was prepared for each chemical, namely, limonene: 100, 89.9, 79.9, 40, 20, 10.6, 5.1, 2.5, 1.7%; n-nonane: 100, 89.9, 80, 40, 20, 10, 5, 2.5, 1%; and D4: 100, 95.5, 89.9, 79.9, 40, 19.9, 10, 4.9, 2.5%. Two mL of each concentration solution was transferred into a 20-mL autosampler vial, which was then closed air tight and left in darkness at 20 ± 1 $^{\circ}\text{C}$ for 24 h for equilibration. Fully automated headspace sampling was performed on the test vials utilizing an autosampler, and the samples were injected on a GC-MS to measure the chemical concentrations in the vapor phase of the HS-PD system relative to the saturated vapors above the pure test substances. The experiment was performed in triplicate.

We used the pure liquid chemical as analytical standard and as the thermodynamic reference ($a_{\text{saturation}} = 1$). Therefore, the chemical activity (a) in the test system was determined by dividing the headspace measurement of that system (*peak area*) by the headspace measurement above the pure test chemical, after confirming the linearity of the MS by separate measurements.

2.2.4. Headspace GC-MS

168 Headspace samples were initially taken manually and injected on a GC-FID, which however provided
169 insufficient analytical precision. Headspace samples were then taken with a CTC PAL RSI 85 autosampler
170 (CTC Analytics, Zwingen, Switzerland) and injected directly onto an Agilent Technologies GC-MSD system
171 (7890B/5877A GC/MSD). On the autosampler, the samples were equilibrated at 35 °C at intermittent
172 orbital shaking for 10 minutes before 100-μL samples were withdrawn from the headspace of the test vials.
173 All analyses were run with a split ratio of 100:1, a DB-5ms Ultra inert column (60 m × 250 μm × 0.25 μm),
174 the following temperature program: initial: 80 °C, rate: 20 °C/min, final: 210 °C, and in TIC scanning mode
175 with a mass-to-charge range from 40-200 m/z. TIC peak areas of test chemicals were manually integrated
176 (MSD Chemstation, Agilent Technologies Inc.) and used for the determination of chemical activity. To
177 ensure that the measured peak areas throughout the entire concentration range were within the linear
178 range of the MS detector, an additional analysis was performed in duplicate for each test chemical with a
179 split ratio of 10:1 (Figure S1). While this exposure analysis based on headspace GC-MS analysis proved to be
180 simple, effective, and precise, it required still about 40 measurements for each of the three chosen
181 chemicals including the important linearity check of the MS detector.

182 2.3. Toxicity tests

183 2.3.1. Algal growth inhibition test

184 The HS-PD approach was applied to control exposure at and below the saturation level. The test chemicals
185 were α-(+)-pinene, α-(-)-pinene, limonene, n-nonane, n-undecane, and n-tridecane. Six concentrations
186 were prepared for each test chemical (Table S4). The donor solutions and test vials were prepared, and a
187 pre-equilibration was completed as described in Section 2.2.1. The *R. subcapitata* growth inhibition tests
188 were conducted as described earlier⁸. Each treatment, including the oil control (insert with Miglyol oil), was
189 performed in 3 replicates, and the global control (without insert and oil) was performed in 6 replicates. A
190 lipid content of 17-19.5% (w/w, dry weight) has been reported for *R. subcapitata*.^{37,38}

191 2.3.2. Acute springtail lethality test

192 The springtail *F. candida* was cultured in closed Petri dishes containing a cast of a mixture of charcoal and
193 plaster of Paris (1:8). The cast was moistened, and dried yeast was used for feeding. The cultures were
194 maintained at 20 ± 1 °C with a 12:12 h light:dark regime. The springtails had an average fresh weight of
195 approximately 110 µg and were picked randomly from different Petri dishes for toxicity tests. The lipid
196 content of *F. candida* has been reported to be 9.9% dry weight.²⁰ A range of different mass concentrations
197 (%) of each test chemical was prepared in Miglyol oil (Table S4), and the HS-PD was applied to control the
198 exposure in the test vials as described in Section 2.2.1. The test chemicals were α-(+)-pinene, limonene, D4,
199 D5, isooctane, n-nonane, n-dodecane, and isododecane. At test start, 2 µL Milli-Q water was added to the
200 bottom center of the pre-equilibrated test vial to ensure sufficient humidity during the test period. Ten
201 animals were transferred to each vial and most of them were observed on the bottom of the test vial
202 throughout the test. The uptake of test chemicals via the water was negligible due to the small water
203 volume, low aqueous concentrations, and limited water uptake by the animals. Hence, the exposure
204 through air was the only uptake route of test chemicals in this HS-PD system. The springtail lethality test
205 was conducted at 20 ± 1 °C with a 12:12 h light:dark photoperiod. Ten global control vials (with insert only,
206 in this case) and 10 oil control vials (insert with Miglyol oil) were included in the experiment. Three
207 replicates were done for each treatment. After 7 days of exposure, springtails were transferred to clean
208 Petri dishes with a clean and moistened cast of charcoal and plaster of Paris mixture and allowed to recover
209 for 24 h. The lethality was then examined, and springtails were characterized as dead when not able to
210 move any part of their body, after gentle stimulation with a fine brush. In all tests, the lethality of the global
211 controls and oil controls was less than 10%, which confirmed the validity of the springtail tests.

212 2.4. Data treatment

213 2.4.1. Exposure confirmation experiment

214 The relationship between test chemical concentration in the lipid donor and chemical activity was
215 established by plotting the measured chemical activity a as a function of mass concentration of chemical in

216 Miglyol oil (X, %) and fitting the data with a one-phase association exponential model using least-squares
217 regression by GraphPad Prism 5.0 software (GraphPad Software, Inc., USA):

$$218 \quad a = a_{max} \times (1 - e^{-k \times X}) \quad (1)$$

219 2.4.2. Toxicity experiments

220 In the algal growth inhibition test with terpenes, there was no significant difference in algal biomass
221 between the global controls and the oil controls after 72h (Figure S2A). In the test with alkanes, there was a
222 minor but statistically significant difference ($p < 0.05$, Figure S2B). And because the majority of the test
223 solutions were prepared in Miglyol oil, we used the mean growth rate of the oil control samples as the
224 reference for calculating the inhibition caused in the treatments. The growth inhibition was calculated as
225 described earlier⁸. Growth inhibition that was numerically higher than 100% was set to a value of 100%
226 (i.e., full inhibition) for all further data analyses. The concentration of the test chemicals in the donor
227 (C_{Donor}) was calculated as the added mass of chemical relative to the total mass of chemical and Miglyol oil,
228 and concentrations were expressed in units of % (mass:mass) or mmol/kg:

$$229 \quad C_{Donor} = (W_C/MW)/(W_C + W_{oil}) \quad (2)$$

230 Where W_C is the added mass (g) and MW the molecular weight (g mol^{-1}) of the test chemical, and W_{oil} is the
231 added mass (g) of Miglyol oil.

232 The actual chemical activities of limonene, n-nonane, and D4 in toxicity tests were determined by
233 interpolation using their corresponding donor concentrations in toxicity tests and the best fit model
234 determined for each chemical in the exposure confirmation experiment (Equation 1). The chemical activity
235 measurements were due to technical reasons performed at 35 °C, and thus at a temperature somewhat
236 higher than the toxicity test temperature of 20 °C. The higher temperature will lead to higher headspace
237 concentrations but should not have a marked impact on the chemical activity calibration curves.
238 Headspace measurements above donor solutions and pure substance references were done at the same

temperature, and we assume the temperature effect on vaporization of the pure chemical in liquid form to be similar to the temperature effect on the lipid-gas phase transfer of that chemical.

The observed toxicity was linked to concentrations in the donor (C_{Donor} , Equation 2) and also to calculated equilibrium concentrations in membranes ($C_{\text{Membrane}} \rightleftharpoons \text{Donor}$). For the latter, storage lipid to membrane lipid partition coefficients K_{LM} were first calculated as ratio of K_{LW} and K_{MW} (Table 1), and then used to determine “theoretical concentrations in membranes at equilibrium with the lipid donor ($C_{\text{Membrane}} \rightleftharpoons \text{Donor} = C_{\text{Donor}}/K_{\text{LM}}$)”. These conversions were not done for $C_{\text{Donor}} > 30\%$, where headspace GC-MS results indicated non-linear partitioning. For test chemicals without clear toxicity at $C_{\text{Donor}} = 30\%$, no dose-response curves were thus produced.

3. RESULTS AND DISCUSSION

3.1. Confirmation of test chemical exposures

The measurements of headspace concentration and chemical activity were performed for limonene, n-nonane, and octamethylcyclotetrasiloxane (D4), which represent the three groups of test chemicals: terpenes, alkanes, and siloxanes, respectively. The same trend was observed for all three chemicals. At low mass concentrations, the partitioning of test chemicals between Miglyol and air was proportional and thus followed as expected Henry’s law where partition coefficients apply (Figure 1)¹⁸. At higher C_{Donor} of typically 20-30%, the partitioning became non-linear and entered the Raoult’s law regime. At the highest donor concentrations, the chemical activity measurements clearly showed a deviation from ideal Raoult’s law behavior, with chemical activity already reaching unity at mass concentrations below 100% (e.g., 80%) (Figure 1). The best explanation for this deviation is that the tested chemicals were only fully miscible with Miglyol oil until a certain concentration, above which the concentrations in the headspace were controlled only by pure test substance. It was difficult to visually confirm a physical separation of the high concentration donor solutions into two phases, and the headspace concentration measurements were then instrumental for identifying this phenomenon. Additionally, these measurements provided a solid link

263 between mass concentration and chemical activity, which in turn provided a defined exposure metric for
264 the assessment of toxicity. Low standard error of the mean (SEM) values for replicate vials confirmed well-
265 controlled exposure, and the high coefficients of determination (r^2) led to well-defined exposure via
266 interpolation (Figure 1).

267 Analytical confirmation of exposure in toxicity tests is essential for reliable toxicity assessments. For a test
268 system using passive dosing with a dominant donor, it is usually sufficient to perform the exposure
269 confirmation at the end of the test or in a separate test without the test organisms. Indeed, passive dosing
270 with a dominant reservoir of test chemical in the donor can buffer losses during testing^{7,20,39–41}. As shown in
271 Table 1, chemicals with high K_{AW} values distribute predominantly (> 90%) to the headspace of a closed test
272 system without a passive dosing donor. In this scenario, a majority of test chemical thus partitions into the
273 headspace after being introduced into the aqueous test medium^{3,42}, which in aquatic toxicity tests can lead
274 to un-controlled exposure and severe underestimation of toxicity². In a passive dosing test system, the test
275 chemicals are contained predominantly in the donor, e.g., > 95% (Table 1), which then via equilibrium
276 partitioning will buffer any losses in the test medium and thus maintain the exposure. The mass balance
277 calculations demonstrate the much higher test substance masses and concentrations in the headspace
278 compared to the water, which make exposure confirmation via the headspace more straightforward and
279 meaningful. We have in the present study also tried to confirm the exposure in the water, which turned out
280 to be very difficult for VHOCs contained in these small volumes of water. Isooctane was the only test
281 substance with a lipid to air partition coefficient $K_{LA} < 1000$, the only chemical with a donor depletion
282 > 10%, and thus omitted from all further data analysis.

283 To assess whether a test chemical exerts baseline or excess toxicity, external exposure concentrations can
284 be converted to internal exposure metrics such as lipid normalized concentration, calculated equilibrium
285 concentration in lipid, or chemical activity^{20,27,28}. In this regard, the HS-PD from a lipid-based donor has a
286 number of advantages. First, the observed toxicity can be linked directly to the test concentrations in the
287 lipid donor for a simplified baseline toxicity assessment. Second, concentrations in the donor can be

converted to calculated equilibrium concentrations in membranes ($C_{\text{Membrane}} \rightleftharpoons C_{\text{Donor}}$), premised that required storage lipid-membrane lipid partition coefficient values are available,³⁶ and effective concentrations can then be compared directly to the critical membrane concentration of 40-160 mmol/kg. Third, the combination of HS-PD and HS-GCMS allows control and measurement of chemical activity in the test system. Measuring head space concentrations and chemical activity is essential for VHOCs, especially when it is challenging to measure their concentrations in the aqueous medium or in the test organism. The measurement is simple, and one can obtain precise data by utilizing fully automated analytical instruments. This again provides a solid exposure metric to be linked to the observed toxicity.

Figure 1: Chemical activity of limonene (A), n-nonane (B), and octamethylcyclotetrasiloxane D4 (C) measured at different mass concentrations in Miglyol oil (mean $\pm$ SEM, $n = 3$).

3.2. Toxicity test results

The toxic effects of limonene, n-nonane, D4, and D5 on algae and springtail were examined both on a lipid basis and a chemical activity basis (Figure 2 and 3), whereas the toxic effects of other test chemicals were examined on the lipid basis only (Figure S3 and S4). Table S1 summarizes lipid based median effective concentrations (EC-50s) and Table S2 median effective chemical activities (Ea-50s) of limonene, n-nonane, and D4 in toxicity tests with both algae and springtails.

Figure 2A shows the growth inhibition of *R. subcapitata* after 72-h exposure to limonene, n-nonane, D4, and D5 against their concentrations in the lipid donor (Equation 2). The siloxanes exhibited no (by D5) or

307 limited growth inhibition (11% by D4) toward *R. subcapitata* as reported by Trac and colleagues⁸, and thus
308 no EC-50 or Ea-50 values were derived for siloxanes in the algal growth inhibition tests. Figure 2B shows the
309 dose-response curves of limonene and n-nonane on the membrane basis, which is within and slightly above
310 the grey box depicting the concentration range for baseline toxicity. Their EC-50 values were 210 and 130
311 mmol/kg lipid, respectively (Table S1). This indicates that these two chemicals were baseline toxic towards
312 algae and springtails and thus no excess toxicity was observed.

313 Figures 2C and 2D show the springtail lethality after 7-d exposure to limonene, nonane, D4, and D5 against
314 their concentrations in the lipid donor and in the membranes at equilibrium with the donor. On the
315 membrane basis, the EC-50 values were 90, 90, 70, and 70 mmol/kg, respectively (Table S1). For the other
316 test chemicals, the EC-50 values in all toxicity tests with both algae and springtail were within or above the
317 baseline toxicity range (Figure S4 and Table S1). These findings support the hypothesis that these test
318 chemicals did not exert excess toxicity.

319 When assessed on the chemical activity basis, the Ea-50 values for limonene and nonane in the algal
320 growth inhibition tests were 0.25 and 0.40, respectively (Table S1), whereas the Ea-50 values for limonene,
321 nonane, and D4 in the springtail lethality tests were 0.11, 0.30, and 0.42, respectively (Table S1). Recent
322 studies on algal growth inhibition caused by a large number of chemicals have reported that for nonpolar
323 narcotic chemicals baseline toxicity requires 1% of saturation, i.e., chemical activity of 0.01²¹, whereas
324 excess toxicity occurs at chemical activity below 0.001, corresponding to 0.1% of saturation²³. Similar
325 findings have been reported for the springtail *F. candida* regarding the chemical activities required for
326 baseline toxicity²⁰. Again, the toxicity of limonene, n-nonane, D4, and D5 in the present study was observed
327 to initiate above the chemical activity range of 0.01-0.1 for baseline toxicity (Figure 3), thus supporting the
328 absence of excess toxicity by these chemicals.

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330

331 **Figure 2:** Toxicity of limonene, n-nonane, octamethylcyclotetrasiloxane (D4), and
 332 decamethylcyclopentasiloxane (D5) to algae and springtail linked to concentration in lipid donor (A & C)
 333 and concentration in membranes at equilibrium with the donor (B & D) (mean $\pm$ SD, $n = 3$). Data for D4 and
 334 D5 in algal growth inhibition test were from Trac et al.⁸. The grey boxes depict the reported concentration
 335 range of 40 – 160 mmol/kg membrane lipid for baseline toxicity.

336 For each test chemical toxicity was higher in springtail than algae (Table S1 and S2, Figure 2 and 3).
 337 Remarkably, the springtail lethality at saturation was 100% for D4 and about 50% for D5, which is markedly
 338 higher than the corresponding algal growth inhibition of D4 (11% at saturation) and D5 (no inhibition)⁸.
 339 These toxicity differences for the siloxanes between the air-exposed springtail and the water-exposed algae
 340 can be explained by differences in the mass transfer of these chemicals through air and water. Indeed, the

341 bioconcentration kinetics of hydrophobic organic chemicals in aquatic organisms is generally rate limited by
342 molecular diffusion through an unstirred aqueous boundary layer (UBL)⁴³. Mayer and colleagues⁴
343 demonstrated that the diffusive mass transfer of 2-3 ringed PAHs was higher through air than through
344 water. This observation is consistent with the observation by Hanzel and colleagues^{5,6}, who demonstrated
345 that diffusive mass transfer of naphthalene is more efficient through soil interstitial air compared to soil
346 interstitial water, and that air-exposure consequently can drive the naphthalene bioavailability to soil
347 bacteria. Both observations can be explained by a higher product of diffusion coefficient and UBL
348 concentration gradient for 2-3 ringed PAHs in air compared to water. The test chemicals in the present
349 study have much higher air to water partition coefficients (K_{AW}) compared to PAHs. Hence, we expected
350 much higher diffusive mass transfer and thus faster uptake kinetics for air compared to water exposure,
351 which is consistent with the observed presence of toxicity in springtails and absence of toxicity in algae.
352 These siloxanes are highly hydrophobic (Log K_{OW} of 6.98 and 8.09⁴⁴) and rather volatile (Log K_{AW} of 2.74 and
353 3.13⁴⁴). Their high hydrophobicity leads also to high concentration and retention in waste water treatment
354 plant (WWTP) sludge, soils, and sediments, and the high K_{AW} values make air-exposure crucial for soil
355 dwelling organisms, particularly in WWTP sludge amended soil. The combination of headspace passive
356 dosing and the springtail lethality test can then be a simple and efficient way to link such soil air-exposure
357 to toxicological endpoints.

359 **Figure 3:** Toxicity of limonene, nonane, and octamethyltetracyclosiloxane (D4) to algae (A) and springtail (B)
360 linked to chemical activity (mean $\pm$ SD, $n = 3$). Data for D4 in algal growth inhibition test were from Trac et
361 al. (mean $\pm$ SD, $n = 6$)⁸. The grey boxes depict the reported chemical activity range of 0.01-0.1 for baseline
362 toxicity.

363 The HS-PD method developed in the present study is an effective method that enables the dose-response
364 testing of liquid VHOCs and provides also an efficient way to assess their inherent toxicity by linking toxicity
365 to estimated equilibrium concentrations in membranes. We expect the method to be applicable to
366 chemicals with a sufficiently high air-water partition coefficient to allow equilibration via the headspace. All
367 test chemicals in the present study have $K_{AW} > 1$ L/L, while other headspace passive dosing studies included
368 semivolatile organic chemicals with lower K_{AW} ³¹⁻³³. Further, the test chemicals need as a basic rule $K_{LW} >$
369 1000 L/L and $K_{LA} > 1000$ L/L in order to avoid donor depletion. Headspace passive dosing seems also suited
370 for chronic and even multi-generation ecotoxicity testing with small aquatic and terrestrial animals^{41,45},
371 since it circumvents biofilm formation on the passive dosing donor (i.e., no direct contact with medium)
372 during extended test durations. Such chronic and multi-generation studies are particularly needed for
373 highly hydrophobic chemicals that are prone to slow bioconcentration kinetics, even for the small test
374 organisms used in the present study.

375 The loading of the passive dosing donor is one of the most time consuming and critical steps in passive
376 dosing. A procedural novelty in the present work is the preparation of the donor by simply adding the liquid
377 test chemical to a liquid donor lipid. This allows setting up the test systems for a reasonably large number
378 of chemicals, treatments and replicates to be tested without significant delay. A main limitation of using a
379 lipid donor has been that we could not find a lipid that is practical (i.e., high capacity, low viscosity liquid,
380 clean, and good commercial availability) and at the same time is the optimal surrogate for the phospholipid
381 membrane. The Miglyol oil used in the present study was practical and provided excellent passive dosing
382 performance, and the subsequent conversions using estimated K_{LM} values were also found a practical and
383 effective way to correct for partitioning differences between storage and membrane lipids.

384 The new combination of exposure control and analytical exposure confirmation for VHOCs can provide
385 sound toxicity data, which now can be further assessed, applied to models, and used in risk assessment.
386 Specifically, the headspace concentration measurements above the lipid donor provided an analytical
387 calibration between equilibrium concentrations in lipids and the thermodynamic chemical activity (Figure
388 1). In the present study, both exposure metrics were used to assess VHOC toxicity and led to the same
389 results and conclusions.

390 **ASSOCIATED CONTENT**

391 **Supporting information**

392 Additional figures for the chemical activity measurements, algal growth in the controls in algal inhibition
393 tests, toxicity of other test chemicals assessed on the lipid basis, and additional tables for toxicity values
394 and dose-response data.

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