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**HEADSPACE PASSIVE DOSING OF VOLATILE HYDROPHOBIC CHEMICALS – AQUATIC TOXICITY TESTING
EXACTLY AT THE SATURATION LEVEL**

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Abstract

It is challenging to conduct aquatic tests with highly hydrophobic and volatile chemicals while avoiding substantial sorptive and evaporative losses. A simple and versatile headspace passive dosing (HS-PD) method was thus developed for such chemicals: The pure liquid test chemical was added to a glass insert, which was then placed with the open end in the headspace of a closed test system containing aqueous test medium. The test chemical served as the dominating partitioning donor for establishing and maintaining maximum exposure levels in the headspace and aqueous solution, without direct contact between the donor and the test medium. The HS-PD method was cross validated against passive dosing with a saturated silicone elastomer, using headspace gas chromatography as analytical instrument and saturated vapors as reference. The HS-PD method was then applied to control the exposure in algal growth inhibition tests with the green algae *Raphidocelis subcapitata*. The model chemicals were C9-C14 n-alkanes and the cyclic volatile methyl siloxanes octamethyltetracyclosiloxane (D4) and decamethylpentacyclosiloxane (D5). Growth rate inhibition at the solubility limit was 100 % for C9-C13 n-alkanes and 53 ± 31 % (95% CI) for tetradecane. A moderate inhibition of 11 ± 4 % (95% CI) was observed for D4, whereas no inhibition was observed for D5. The present study introduces an effective method for aquatic toxicity testing of a difficult-to-test group of chemicals and provides an improved experimental basis for investigating toxicity cut-offs.

Keywords: algal growth inhibition, solubility limit, high Henry's constants, n-alkanes, siloxanes, toxicity cut-offs.

32 **Graphical abstract**

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35 **Highlights**

- 36 • Passive dosing via the headspace established and maintained constant exposure.
- 37 • Headspace passive dosing was cross validated by silicone rod passive dosing.
- 38 • Aquatic toxicity testing of volatile hydrophobic chemicals exactly at saturation.
- 39 • Algal growth inhibition limit tests showed toxicity of alkanes and D₄, but not D₅.

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1. Introduction

The environmental risk assessment of organic chemicals requires proper aquatic toxicity testing, which for some chemicals is straightforward, whereas it for other chemicals is associated with substantial scientific, technical, and analytical challenges (OECD, 2000; Rufli et al., 1998). The quality of the risk assessment of such difficult-to-test chemicals will then highly depend on whether the available test methodologies are fit for the purpose. There are several physical-chemical properties that can make chemicals difficult to test, including limited stability, light attenuation (color), pH sensitive speciation, and surface active properties (OECD, 2000; Rufli et al., 1998). However, two of the most often encountered properties that lead to experimental difficulties are high hydrophobicity and high volatility. High hydrophobicity is generally indicated at octanol to water partition coefficients (K_{ow}) above 10^5 (i.e., $\text{Log } K_{ow} > 5$) and at aqueous solubility in the ng- μg per liter range. Highly hydrophobic chemicals can be difficult (1) to introduce into the test medium, (2) to keep at constant concentrations during the test, and (3) to test up to the solubility limit while avoiding the presence of pure chemical in the test medium. High volatility is in aquatic toxicity testing best indicated by high air to water partition coefficients ($K_{air,water}$) (unit less Henry's constant, L L^{-1}). Chemicals with $K_{air,water} > 1$ are particularly difficult to test since they are prone to substantial evaporative losses, even when tested in closed test systems, due to their high tendency to partition into the headspace (Birch et al., 2017; Mayer et al., 2000). There are about 3900 chemicals with $K_{air,water} > 1$ and $\text{Log } K_{ow} > 5$ registered in the Danish QSAR database (Danish (Q)SAR Database, 2018), which are difficult to test with currently available methods.

During the last two decades, a range of passive dosing techniques have been developed and applied for controlling the exposure to hydrophobic organic chemicals (HOCs) in various aquatic toxicity tests (Bragin et al., 2016; Butler et al., 2013; Mayer et al., 1999; Seiler et al., 2014; Smith et al., 2010a, 2010b; Stibany et al., 2017). In passive dosing, a partitioning donor is loaded with the test chemical(s) and applied to establish well-defined concentrations at the start of the test, and then to maintain constant concentrations during

the test (Smith et al., 2010a). While passive dosing has been successfully applied in many studies, there are still several challenges and a need for further developments: (1) Passive dosing has mainly been applied to solid HOCs (Mayer and Holmstrup, 2008; Smith et al., 2013, 2010a) and to liquid HOCs with moderate volatility (Stibany et al., 2017), and it is thus crucial to extend this work to liquid HOCs with high volatility. (2) Many passive dosing formats require substantial preparative work prior to the toxicity tests (Smith et al., 2010a), and time efficient passive dosing methods suited for high throughput testing are urgently needed (Vergauwen et al., 2015).

Recently, a new passive dosing method was developed for testing liquid HOCs exactly at the solubility limit (i.e., limit testing) (Stibany et al., 2017). This method employed a silicone O-ring, which was first loaded to saturation by direct immersion in the pure liquid test chemical. The saturated silicone was then placed in the test medium for controlling the exposure exactly at the saturation level. This approach is simple, practical, and leads also to reproducible toxicity data. Although not observed in the specific study by Stibany et al. (2017), direct contact between a saturated donor and test medium could, in worst case, lead to micro droplets entering the test medium, which would then compromise the interpretation of the observed effects. This calls for novel passive dosing methods that exclude the direct contact between the passive dosing donor and the test medium.

The present study introduces a new, simple, and versatile passive dosing method for aquatic toxicity testing of volatile HOCs (i.e., liquid chemicals with high Henry's constants), with no direct contact between the donor and the test medium. This new passive dosing approach is operated via the headspace (i.e., headspace passive dosing, HS-PD): The test chemical itself acts as the donor that quickly saturates the headspace through vaporization, which then saturates the aqueous test medium by air water equilibrium partitioning. The donor is easily administrated by adding the pure test chemical to an insert, which is then placed directly in the closed test vial containing aqueous test medium and headspace (see the graphical abstract for visualization of the system). Through the vapor phase, the test chemical is introduced into the

test medium as individual molecules, thus avoiding the introduction of micro droplets. However, re-condensation of test chemical on the inner surfaces of the test system cannot be ruled out when operating the method exactly at the saturation level. Testing just below the saturation limit should therefore be included to elaborate this condensation effect.

The careful control and measurement of exposure in environmental testing have implications well beyond the technical level. In fact, several passive dosing studies have already shown how improved exposure control can lead to fundamental new results and also provide a better understanding of toxicity (Schmidt et al., 2013; Seiler et al., 2014; Stibany et al., 2017), biodegradation (Birch et al., 2018, 2017; Smith et al., 2012), toxicity cut-offs (Mayer and Holmstrup, 2008), and mixture toxicity (Bjergager et al., 2017; Smith et al., 2013). On the scientific level, the present study aimed to determine the aquatic toxicity of n-alkanes and cyclic volatile methyl siloxanes (cVMS). N-alkanes are significant constituents of petroleum products (De Graaff et al., 2012) and used as intermediates in the production of surfactants and detergents (American Chemistry Council n-Alkane VCCEP Consortium, 2004). The n-alkanes with the number of carbon atoms ranging from 9 – 14 (C9 – C14) were selected as model chemicals because they have a high Henry's constant, their toxicity to aquatic organisms is still in question, and they comprise a wide range of carbon chain length, which makes them suitable for studying cut-offs in toxicity. The cVMS D4 (octamethylcyclotetrasiloxane) and D5 (decamethylcyclopentasiloxane) were also selected, since they are widely used in personal care products as well as industrial applications (Fairbrother et al., 2015; Fairbrother and Woodburn, 2016). They are chemicals of emerging concern and have become the subject of interest for both scientific and regulatory scrutiny, since they have been detected and monitored in air, water, sediments, and biota (Gobas et al., 2015; Mackay et al., 2015a). Their high volatility and low solubility present challenges for assessing the ecological risks of these chemicals to aquatic systems (Fairbrother and Woodburn, 2016; Mackay et al., 2015b).

Therefore, the aim of the present study is twofold: (1) to develop and validate a simple and versatile headspace passive dosing method for testing liquid chemicals with high Henry's constants, which are difficult to test with currently available methods, and (2) to apply the method to the green algae *Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata* and *Selenastrum capricornutum*) growth inhibition tests with n-alkanes and siloxanes at, and just below, their saturation levels to investigate the condensation effects and better understand their aquatic toxicity. The hypotheses of the study are: (1) The headspace passive dosing approach can provide exposure exactly at the saturation limit by equilibrium partitioning between the liquid test chemical and the aqueous test medium via the headspace, and (2) n-alkanes C9 – C14 and siloxanes D4 and D5 can exert toxicity to the algae *R. subcapitata* when tested at the maximum exposure level, i.e., at chemical activity of unity (Reichenberg and Mayer, 2006).

2. Materials and methods

2.1. Materials and chemicals

Clear 20-mL glass vials (ND18, 75.5 × 22.5 mm and ND24, 57 × 27.5 mm), screw caps with Teflon-lined (PTFE) septa, and 0.3-mL glass inserts were purchased from Mikrolab Aarhus A/S, Denmark. Pyrex® fiber glass wool (8 µm per strand) was from Sigma-Aldrich (Denmark). Translucent silicone rods with a diameter of 3 mm were custom made by Altec Products Ltd (United Kingdom) and used as partitioning donors in the silicone rod passive dosing system (see Section 2.3.). The test chemicals were nonane (≥99%, Fluka AG, Buchs SG, Switzerland), decane (≥99%, Sigma-Aldrich, Denmark), undecane (≥99%, Sigma-Aldrich Chemie, Germany), dodecane (>99%, TCI Chemicals, Belgium), tridecane (≥99%, Fluka AG, Switzerland), tetradecane (≥99%, Fluka AG, Switzerland), octamethylcyclotetrasiloxane (D4, 98%, Sigma-Aldrich, Denmark), and decamethylcyclopentasiloxane (D5, 97%, Sigma-Aldrich, Denmark). Miglyol 812 oil was used for diluting the pure test chemicals and was from Cremer Oleo GmbH & Co. KG, Germany. Ethanol and acetone were purchased from VWR (HPLC grade, VWR International GmbH, Germany). Milli-Q water (LaboStar™ 1-DI

137 ultra-pure water system, Evoqua water technologies, Germany) was used in algal test medium and for
138 washing the silicone rods. Lint-free tissues (Assistent, Germany) were used for drying the silicone rods.
139 Isotone (Coulter isotone II diluent, Beckman Coulter GmbH, Germany) was used as diluent for coulter
140 counting to determine algal cell densities in the pre-cultures.

141 2.2. Development of the headspace passive dosing method

142 The headspace passive dosing (HS-PD) approach was developed to enable testing of chemicals with high K_{ow}
143 and high $K_{air,water}$ while avoiding the introduction of pure phase test chemical into the test medium and
144 minimizing the preparatory work prior to toxicity testing. Specifically, 0.1 mL of a liquid test chemical was
145 simply added to a 0.3-mL glass insert, which was then placed in a 20-mL ND24 glass vial (test vial)
146 containing 4 mL of algal test medium (see the graphical abstract for visualization of the system). The pure
147 test chemical thus acted as the dominating partitioning donor controlling the exposure in the headspace as
148 well as in the aqueous solution by equilibrium partitioning. Glass wool was placed in the inserts to increase
149 the surface area and thereby facilitate the partitioning process between the pure phase and the headspace.
150 The test vials were sealed tight, and the complete system was then shaken (200 rpm, 10 mm orbit) for 24 h
151 at 20 ± 1 °C to ensure equilibrium between the donor, headspace, and test medium at test start (pre-
152 equilibration). The pre-equilibration was performed in the dark to avoid photodecomposition.

153 Exposure of a given test chemical at its saturation level in the HS-PD system was confirmed by headspace
154 gas chromatography with flame ionization detector (headspace GC-FID) using saturated vapor over the
155 pure liquid as reference. The saturated vapor of a test chemical was simply prepared by adding 1 mL of the
156 chemical into a test vial and subsequently shaking the vial (200 rpm, 10 mm orbit) for 24 h at 20 ± 1 °C in
157 the dark.

158 2.3. Cross validation by the silicone rod passive dosing method

Passive dosing using a saturated silicone polymer is a well-established and reproducible approach for controlling the exposure in aquatic toxicity testing of hydrophobic organic chemicals (HOCs) exactly at the saturation limit (Smith et al., 2010a; Stibany et al., 2017). This approach was therefore used to cross validate the HS-PD approach. For preparing the silicone donors, a long silicone rod was first soaked in excess ethanol and shaken overnight to remove dust and impurities. The rod was then rinsed three times with excess Milli-Q water during a period of > 24 h to remove ethanol and dried with lint-free tissues. Then, the rod was cut into pieces of approximately 4 cm and weighed. The loading was done in closed 20-mL ND18 glass vials, where the silicone rods were immersed in excess pure test chemical for at least 24 h. Preliminary experiments showed that 24 h is sufficient for the test chemicals to reach saturation in silicone rods, indicated by no additional weight gain in the loaded rods (results not shown). The resulting saturated silicone rods were rinsed three times with Milli-Q water, dried with lint-free tissues, re-weighed, and eventually used for establishing and controlling the exposure in the test system. They were placed standing upright in the test vials containing 4 mL of algal test medium (see Figure S1 for visualization of the system). In this way, the exposure in the aqueous medium was controlled by equilibrium partitioning via the headspace and by direct passive dosing from the silicone rod. The test vials were then shaken (200 rpm, 10 mm orbit) for 24 h for pre-equilibration. Both the loading and pre-equilibration were performed at 20 ± 1 °C in the dark. The exposure levels in this passive dosing system were also measured using headspace GC-FID and compared to the results from the HS-PD method and the saturated vapors over the pure liquids (see Section 2.2).

2.4. GC-FID analysis

Thermo Scientific™ Trace™ 1300 GC-FID was used for quantifying test chemicals. A 50-μL air sample was taken from the headspace of test vials using a Hamilton 50-μL gas tight syringe and manually injected. The syringe was cleaned with ethanol vapor and flushed with air for drying between samplings, and none of the test compounds was detected in control injections. The instrument was operated with a Trace™ TR-5 0.53

mm, 1.5 μm , 30 m column (Thermo Scientific, Italy), an injection temperature of 150 $^{\circ}\text{C}$ with a split ratio of 3:1 and a helium carrier flow rate of 3 mL min^{-1} . The temperature was initially set to 40 $^{\circ}\text{C}$, held for 2 min, increased by 25 $^{\circ}\text{C min}^{-1}$ up to 180 $^{\circ}\text{C}$ and finally held for 1 min. Peak area integration was accurately performed with ChromeleonTM 7.2 CDS software (Thermo Scientific).

2.5. Mass distribution calculations

The mass distribution of test chemicals in the two passive dosing systems, i.e., headspace and silicone rod, was estimated to illustrate the large buffer capacity of the donor and the high partitioning of the test chemicals to the headspace compared to the aqueous phase. The test systems consisted of the following compartments: a partitioning donor, a headspace, and an aqueous medium when assuming negligible adsorption to the glass and PTFE materials. The mass of test chemical in the donor phase of the HS-PD system was calculated based on the volume (i.e., 0.1 mL) and relative density of a given liquid, and the loaded mass of the silicone rod was calculated as the weight differences before and after loading with test chemicals. The mass of test substance in the headspace (m_{HS} , g) was calculated based on the ideal gas law:

$$m_{HS} = (p \times M \times V_{HS}) / (R \times T)$$

where p is vapor pressure (Pa), M is molar mass (g mol^{-1}), V_{HS} is the headspace volume (estimated to be 15.1 mL, considering 0.9 mL is taken up by the insert or silicone rod), R is universal gas constant ($\text{Pa m}^3 \text{mol}^{-1} \text{K}^{-1}$), and T is absolute temperature (298 K). The water solubility values of the test substances were used for calculating the masses in the test medium. Physical-chemical property data were obtained from Environmental Risk Assessment reports (Brooke et al., 2009a, 2009b) for siloxanes and from Handbook of Physical-Chemical Properties and Environmental Fate for Organic Chemicals (Mackay et al., 2006) for n-alkanes.

2.6. Algal growth inhibition tests at the solubility limit

205 The *Raphidocelis subcapitata* growth inhibition test was based on the OECD guideline 201 (OECD, 2011). A
 206 small adjustment, i.e., a 150 mg L⁻¹ NaHCO₃ enrichment of the algal medium, was made to buffer pH
 207 changes in the closed test system (Mayer et al., 2000). Pre-cultures were maintained for a week before test
 208 start with a renewal interval of every other day. The algal cell density was determined using the Coulter®
 209 Particle Counter Z1 (Beckman Coulter GmbH, Germany).

210 First, the HS-PD approach was applied to maintain the exposure to the test chemical exactly at the
 211 saturation limit. Pre-equilibration was completed prior to test start to ensure equilibrium of test chemicals
 212 among all phases in the test system, as described in Section 2.2. Second, an additional treatment with
 213 approximately 80% pure test chemical was included to investigate the condensation effect in the test
 214 system (i.e., the toxic effect that might have been caused by condensed pure test chemical in the system).
 215 The donor phase of this treatment was prepared by mixing the pure test chemical with Miglyol oil with a
 216 ratio of 4:1 (w:w). Therefore, oil control samples were included and contained 0.1 mL Miglyol oil only in the
 217 inserts. The global control samples, which were used to check the validity of the test as well as a reference
 218 to calculate growth inhibition, contained 4 mL of algal medium only.

219 All tests were conducted as described by Stibany and colleagues (Stibany et al., 2017). Briefly, the
 220 pre-equilibrated test vials were opened (one vial at a time) and algal pre-culture was added to reach an
 221 initial algal density in each vial of 10⁴ cells mL⁻¹. Then, the test vials were sealed and 175 µL CO₂ was added
 222 to the headspace (using a syringe) to ensure sufficient supply of CO₂ during the closed test (Halling-
 223 Sørensen et al., 1996). Test vials were shaken (300 rpm, 3 mm orbit) at 20 ± 1 °C under continuous
 224 illumination from beneath. The light intensity was 123 ± 3 µmol m⁻² s⁻¹ photosynthetically active range
 225 (PAR) photons. After 24, 48, and 72 h, algal biomass was measured by *in vitro* chlorophyll fluorescence
 226 (Mayer et al., 1997) and used in the calculation of the specific growth rate (μ , d⁻¹):

$$\mu = (\ln(N_t) - \ln(N_0))/t$$

where N_t is the algal biomass at sampling time t (d) and N_0 is algal biomass at test start. The toxic effect was calculated as the percent reduction of growth rate (I , %) relative to the control (without insert) for each treatment replicate:

$$I = (1 - \mu_{treatment}/\mu_{control}) \times 100$$

In accordance with the ISO 8692 guideline (DIN, 2012), a minimum growth rate of 1.4 day^{-1} in the controls was used as validity criterion for the tests.

For n-alkanes, the algal growth inhibition test was performed once with six control replicates and three sample replicates for each chemical. For D4 and D5, three separate tests were performed with the same replicate setup (except the first test with just three control replicates). The validity criterion was not met in the third test with the siloxanes, and the average results of the first and second tests were therefore used.

2.7. Data treatment

One-way analysis of variance (ANOVA) was conducted to test significant differences in exposure levels between the HS-PD system, the silicone rod system, and the saturated vapors. Pairwise multiple comparisons were performed following the one-way ANOVA test using Holm-Sidak's method in case there were differences. Dunnett's t-test was conducted to test significant differences in algal growth rates between treatments and global controls. Data were checked for normal distribution and homogeneity of variances by Kolmogorov-Smirnov's test and Levene's test, respectively. All statistical analyses were performed using SigmaPlot 11.0 (Systat Software Inc., California, USA) with a significance level of 0.05 (i.e., $p < 0.05$). For the presentation of algal growth inhibition caused by n-alkanes, data from treatments that showed more than 100 % inhibition were set to 100 % and then used for analysis.

3. Results and discussion

3.1. Headspace passive dosing and cross validation by silicone rod passive dosing

249 After 24 h of equilibration, measurements of n-alkanes and siloxanes in the headspace of both passive
250 dosing systems corresponded well to the reference measurements of the saturated vapors over the pure
251 liquids (Figure 1). There were some minor but statistically significant differences, which we attribute to
252 technical issues and particularly a minor temperature difference between the equilibration period and
253 waiting time prior to manual injection for GC-FID analysis. For most test substances, there was no
254 statistically significant difference in the measured exposure levels between the two passive dosing systems.
255 The only real exception was C9 with measurements in the silicone rod system being lower compared to the
256 measurements in the HS-PD system as well as to the reference system (Figure 1A). Differences in exposure
257 in cases of C10 and C11 were statistically significant between the two passive dosing methods and the
258 reference system, but less than 30 %. Overall, the analytical results confirmed that both passive dosing
259 systems were able to establish exposure of the test chemicals at their saturation level within 24 h
260 equilibration. Furthermore, results from the two passive dosing methods cross validated each other based
261 on the good agreement between measurements in the headspace. This makes the two methods
262 compatible and well aligned.

It has been shown in previous studies that mass transfer of volatile hydrophobic organic chemicals (HOCs) between water and headspace in 2-20 mL closed test systems is fast (Mayer et al., 2000; Zhang and Pawliszyn, 1993). Indeed, this is the reason for the very large and fast evaporative losses of volatile organic test substances in, for instances, algal growth inhibition testing (Mayer et al., 2000), and the basis for the analytical technique Headspace Solid-Phase Microextraction (HS-SPME) (Zhang and Pawliszyn, 1993). In our HS-PD system, fast mass transfer of test chemical into the aqueous medium takes place via the headspace and through vaporization of test chemical from the donor. At equilibrium, aqueous concentrations are at maximum solubility when headspace concentrations are at the saturation limit. Therefore, measuring test chemicals in the headspace of the two passive dosing systems and relating these results to saturated vapor references was a fast and precise approach to confirm the exposure at saturation.

3.2. Mass distribution

The mass distribution calculations illustrate that chemicals with high Henry's constants distribute more to the headspace than to the aqueous phase (Table 1). Specifically, the amount of n-alkane was about 70 to 3400 times (for nonane and undecane, respectively) higher in the headspace than in the test medium, while the differences were about 1100 times for the siloxanes. This implies very high evaporative losses of such chemicals to the headspace, and thus a dramatic decrease of aqueous medium concentrations when applying methods not optimized for chemicals with high Henry's constants (Birch et al., 2017; Mayer et al., 2000). The mass distribution calculation also emphasizes that the passive dosing donor, at equilibrium, carries at least 99.4% (for nonane) of the test chemical in the test system, which means that the donor phase is truly the dominating partitioning source for controlling the concentration in the test medium. This buffer capacity is highly sufficient to replenish the aqueous medium with test chemicals in case of losses and can therefore maintain constant exposure of the test chemicals throughout the 72-h test period.

Table 1: Mass distribution of n-alkanes and cyclic volatile methyl siloxanes in the silicone rod (Rod) and headspace (HS) passive dosing systems.

	Rod	HS	Rod	HS	Rod	HS
	nonane		decane		Undecane	
Donor (%)	99.87	99.35	99.95	99.78	99.98	99.93
Headspace (%)	0.13	0.64	0.048	0.22	0.018	0.074
Medium (%)	1.9e-03	9.5e-03	1.8e-05	8.3e-05	5.3e-06	2.2e-05
	dodecane		tridecane		Tetradecane	
Donor (%)	99.99	99.97	99.997	99.99	99.999	99.997
Headspace (%)	7.0e-03	0.025	2.7e-03	8.5e-03	1.1e-03	2.9e-03
Medium (%)	5.5e-06	2.0e-05	1.7e-06	5.5e-06	6.7e-07	1.7e-06
	D4		D5			
Donor (%)	99.95	99.75	99.98	99.92		
Headspace (%)	0.048	0.25	0.016	0.079		
Medium (%)	4.4e-05	2.3e-04	1.4e-05	7.1e-05		

292

293 3.3. Algal growth inhibition tests at the solubility limit

294 When tested at the saturation level, all n-alkanes caused full inhibition of the algal growth rate, except for
 295 tetradecane, causing 53 ± 31 % (95% CI) inhibition (Figure 2). Similarly, full algal growth rate inhibition by
 296 C9-C13 was also observed when the test chemicals were tested at 80 % of the saturation level. The toxic
 297 effect observed at the 80 % saturation level thus confirmed that the algal growth inhibition was caused by
 298 the freely dissolved molecules and not by potential artifacts of condensed micro droplets of the test
 299 chemicals in the test system. The lower inhibition by tetradecane indicated a cut-off point in toxicity for

alkanes with longer chain length. For siloxanes, D4 caused moderate growth rate inhibition, i.e., $11 \pm 4\%$ (95% CI), whereas D5 did not inhibit the algal growth (Figure 2).

302

Figure 2: Algal growth inhibition after 72 h caused by n-alkanes (mean $\pm$ 95% CI, n = 3 replicates) and siloxanes (mean $\pm$ 95% CI, n = 6 replicates) at the saturation and 80 % saturation levels. Inhibition results from two individual tests for the siloxanes are shown in the supporting information (Figures S2 and S3).

D4 and D5 were less toxic than expected (second hypothesis), since they exerted no or only limited toxicity even when tested at the solubility limit (i.e., chemical activity of unity). To the contrary, tetradecane exerted approximately 50% growth inhibition at the solubility limit, despite its Log K_{ow} of 8.0 (Mackay et al., 2006) being well above the reported hydrophobicity cut-off for baseline toxicity at Log K_{ow} of 6 (Könemann, 1981). A similar finding was recently observed for dodecylbenzene (DDB) with a Log K_{ow} of 8.65, which was found to be moderately toxic towards algae when tested at the solubility limit (Stibany et al., 2017). These observations are in good agreement with previous studies on the baseline toxicity of highly HOCs (Mayer and Reichenberg, 2006) and on applying bioconcentration kinetics for the prioritization of aquatic toxicity testing of highly HOCs (Kwon et al., 2016).

315 The toxicity of D4 and D5 to aquatic organisms is of particular interest for regulatory scrutiny. Regulatory
316 assessment of these chemicals has been conducted by many authorities, including United Kingdom
317 Environment Agency (Brooke et al., 2009b, 2009a), Environment Canada and Health Canada (Environment
318 Canada and Health Canada, 2008a, 2008b), and Danish Environmental Protection Agency (Danish EPA,
319 2014). The assessment of D5 has been controversial, as D5 was initially considered to be toxic but was later
320 reported not to pose a danger to the environment (Mackay et al., 2015a). Within the regulatory framework,
321 the observations of the present study suggest that D5 had neither acute nor chronic effects on algal growth
322 at the maximum exposure level. D4 exerted a growth rate inhibition of 11 % when tested at the saturation
323 level, which is very similar to the recently tested dodecylbenzene and can be interpreted as a moderate
324 chronic toxicity to algae (Stibany et al., 2017).

325 From the physical-chemical point of view, the present study demonstrates a simple “plug-and-play”
326 approach to test liquid HOCs with high Henry’s constants. High hydrophobicity and volatility are two of the
327 physical-chemical properties that make a chemical difficult to test (OECD, 2000). However, these properties
328 were utilized in the HS-PD approach to enable simple, practical, and also more efficient toxicity testing of
329 such difficult-to-test chemicals. In addition, the introduction of dissolved molecules via the headspace helps
330 avoid the formation of pure chemicals in the test medium. The preparative work for the partitioning donor
331 was also vastly reduced compared to other passive dosing approaches. For instance, in the passive dosing
332 approaches using silicone polymer there are several steps for preparing the donor, which involve: (1) the
333 selection and dimensioning of the donor material to ensure negligible depletion, (2) the careful cleaning of
334 the donor material prior to loading, and (3) the loading of test substances into the donor material (Butler et
335 al., 2013; Engraff et al., 2011; Schmidt et al., 2013; Seiler et al., 2014; Smith et al., 2013). Hence, the simple
336 preparation of the donor also made the HS-PD approach more versatile by allowing testing both at, and just
337 below, the saturation level. This was easily done without any significant effort by diluting the pure
338 chemicals with Miglyol oil to obtain the 80 % saturation level.

Furthermore, this approach can be applied to other species including fish and invertebrates. However, one should then ensure that (1) the closed test vessel does not lead to significant changes in test conditions (i.e., pH, oxygen content, light intensity, etc.) and also comply with the respective test guidelines, (2) factors that help achieve quick equilibration of test chemicals in the test system should be considered, for example, shaking of test vial and/or increasing surface areas of both test medium and pure test chemical, and (3) the amount of the pure test chemical should be sufficient compared to the amount in the test medium to ensure non-depleted source of test chemical for passive dosing. Further work is thus needed to adapt, optimize, and validate the HS-PD approach for the toxicity testing with other species.

Volatile chemicals can evaporate before and after entering natural aquatic environments, and one might thus ask whether aquatic toxicity testing of these chemicals is necessary. Volatile HOCs are not only prone to evaporation but they also bind to dissolved organic matter and sorb to sludge and sediment. These binding and sorption interactions can then keep volatile HOCs within the aquatic environment for a longer period of time (Panagopoulos and MacLeod, 2018), which poses potential risks to the organisms living in this compartment. It is therefore essential to have the possibility for assessing the environmental impact of such difficult-to-test chemicals and suitable ecotoxicological methods are strongly needed. The two passive dosing methods allow “limit testing at saturation” within a large applicability domain with regards to hydrophobicity and volatility. These approaches can now be used for simplifying and improving the regulatory toxicity testing of volatile hydrophobic organic chemicals, and also for more dedicated studies exploring toxicity cut-off phenomena and challenging existing toxicity cut-off theories.

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