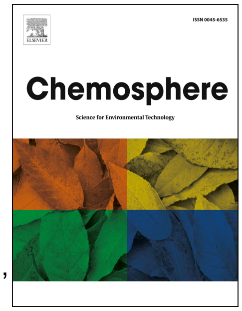

Assessing Toxicity of Hydrophobic Aliphatic and Monoaromatic Hydrocarbons …

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Assessing Toxicity of Hydrophobic Aliphatic and Monoaromatic Hydrocarbons at the Solubility Limit using Novel Dosing Methods

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ABSTRACT

Reliable delineation of aquatic toxicity cut-offs for poorly soluble hydrocarbons is lacking. In this study, vapor and passive dosing methods were applied in limit tests with algae and daphnids to evaluate the presence or absence of chronic effects at exposures corresponding to the water solubility for representative hydrocarbons from five structural classes: branched alkanes, mono, di, and polynaphthenic (cyclic) alkanes and monoaromatic naphthenic hydrocarbons (MANHs). Algal growth rate and daphnid immobilization, growth and reproduction served as the chronic endpoints investigated. Results indicated that the dosing methods applied were effective for maintaining mean measured exposure concentrations within a factor of two or higher of the measured water solubility of the substances investigated. Chronic effects were not observed for hydrocarbons with an aqueous solubility below approximately 5 µg/L. This solubility cut-off corresponds to structures consisting of 13-14 carbons for branched and cyclic alkanes and 16-18 carbons for MANHs. These data support reliable hazard and risk evaluation of hydrocarbon classes that comprise petroleum substances and the methods described have broad applicability for establishing empirical solubility cut-offs for other classes of hydrophobic substances. Future work is needed to understand the role of biotransformation in the observed presence or absence of toxicity in chronic tests.

Key Words: chronic effects, toxicity, hydrocarbons, aqueous solubility, chemical activity, cut-offs

INTRODUCTION:

Substance-specific information on aquatic toxicity is essential for chemicals management priority setting, environmental hazard classification and risk assessment. A commonly observed trend in reported aquatic toxicity data collected across a homologous series of organic compounds is that toxicity increases with increasing hydrophobicity and decreasing solubility of the homologs until a toxicity-cut off is reached (Abernathy et al. 1988; Donkin et al. 1991; Hulzebos et al. 1993; Parkerton & Konkel, 2000; Sverdrup et al. 2002; Schaefers et al. 2009). Beyond this point, effects are not observed for more hydrophobic, less soluble compounds. While these trends are generally applicable, the homolog that defines the toxicity boundary for a given substance class can be modulated depending on the organism, effect endpoint, toxicity test duration and exposure conditions considered (Kang et al. 2017).

Three explanations alone or in combination can help explain these experimental observations. First, it is often difficult to deliver, maintain and analytically confirm exposures of hydrophobic test substances at the corresponding solubility limit. The challenge of exposing test organisms to a maximal upper limit concentration throughout the test is most pronounced for substances that in addition to being poorly soluble are also susceptible to various loss processes (e.g. volatilization, degradation) that can occur during routine toxicity tests (Rogerson et al. 1983; Smith et al. 2010, Niehus et al. 2018). Addressing this challenge requires dosing methods that achieve the solubility limit and compensate for any losses to buffer and thus maintain this concentration during the test. Second, kinetic constraints associated with the design of standard aquatic toxicity tests may preclude sufficient internal concentrations to be achieved in test organisms to express adverse effects within the timeframe of the test (Kwon et al. 2016). The aqueous solubility of a substance sets the maximum concentration gradient that drives diffusive exchange processes (Birch et al, 2019), and low water solubility limits the achievable uptake by test organisms and observable effects within the toxicity test duration. This is particularly problematic for short duration acute tests with larger organisms that exhibit slower uptake rates. This in turn argues that for hydrophobic substances, small test organisms with faster uptake kinetics and chronic tests with longer test duration be selected for hazard assessment. The third aspect is the effect of the melting enthalpy on the solubility of chemicals that are in solid form. The aqueous solubility of solids is the result of both hydrophobicity and the melting costs for transferring the solid substance into a liquid state. The actual solubility of a solid is thus lower than its sub-cooled liquid solubility, and this suppression of the aqueous solubility increases with increasing melting enthalpy and corresponding melting point. The maximum chemical activity that can be achieved for a solid chemical may then be below that needed to invoke toxicity (Mayer and Reichenberg, 2006). This explanation provides a thermodynamic basis to account for observed toxicity cut-offs associated with solids such as polyaromatic hydrocarbons (Rogerson et al. 1983; Mayer et al. 2008; Engraff et al. 2011; Kwon et al. 2016). It is important to note that such solids while non-toxic alone can nevertheless still contribute to effects when present in mixtures (Mayer & Reichenberg, 2006; Smith et al. 2013). However, since liquids can achieve the maximum chemical activity of unity if dosed at the aqueous solubility, a systematic study of the observed toxicity of various hydrophobic liquids provides a logical focus for investigating and delineating toxicity-cutoffs.

The above insights help inform intelligent testing strategies for improved aquatic toxicity evaluation of hydrophobic organic substances. The first recommendation is to integrate recent advances in passive dosing to conduct limit tests at the aqueous solubility of the test substance. This approach offers a particularly pragmatic and cost effective tiered experimental design to determine the presence or absence of toxicity across a homologous series of test substances using a single treatment concentration corresponding to the solubility limit. For homologs that demonstrate inherent hazard at unit activity for liquids or at the maximum achievable chemical activity for solids, subsequent definitive tests for establishing concentration-response relationships can be performed (Stibany et al, 2017a, Stibany et al. 2017b, Trac et al. 2018, 2019). While application of passive dosing methods may involve more effort than traditional dosing procedures, the ability to maintain stable aqueous exposures helps ensure the resulting toxicity data generated are not judged unreliable for regulatory use.

A second recommendation is to select test organisms that exhibit fast uptake rates and incorporate sensitive endpoints. While use of microbial tests, such as Microtox, may seem appealing due to expected rapid uptake rates associated for bacteria and the simplicity of such assays, microbial test endpoints have shown to be less sensitive when testing poorly water soluble substances (Kang et al. 2016; Winding et al, 2019). This is likely due to more than one to two order of magnitude higher critical target lipid body burdens (CTLBBs) reported for these endpoints (Redman et al. 2014) when contrasted to CTLBBs derived for algal and crustacean chronic test endpoints (McGrath et al. 2018). In contrast, the standard short term toxicity test with *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) based on growth inhibition (e.g. EC_{10} or NOEC as endpoint) provides an endpoint that is reported to be at the median of the species sensitivity distribution of estimated chronic critical target lipid body burdens derived using the target lipid model (McGrath et al. 2018). Longer term 21 d *Daphnia magna* or 7 d *Ceriodaphnia dubia* chronic tests enable use of standardized test guidelines with relatively small test organisms, involve even more sensitive and comparable sub-lethal endpoints based on reported CTLBBs and avoiding vertebrate animal testing.

The objective of this study is to apply passive and vapor dosing techniques in algal growth and daphnid toxicity limit studies for hydrocarbons representing branched alkanes, mononaphthenic (saturated monocyclic), dinaphthenic (saturated dicyclic), polynaphthenic (saturated polycyclic) and monoaromatic naphthenics (one aromatic with saturated cyclics) hydrocarbon classes. A tiered approach is applied in which toxicity cut-offs are first established using algal tests which are simpler and less costly to perform. These cut-offs are then confirmed using targeted chronic limit tests with daphnids. This work builds on previous toxicity test data generated for polyaromatic hydrocarbons for these freshwater species and chronic sub-lethal endpoints (Bragin et al. 2017) by further extending passive dosing techniques to other classes of hydrocarbon liquids and solids. Results obtained from this study are compared to relevant literature data and mechanistic modeling predictions for quantifying and understanding the mechanistic basis for observed toxicity cut-offs.

MATERIALS AND METHODS:

Test Substances

Four branched alkanes (2,2,4,6,6, pentamethylheptane, 2,6 dimethyldecane, 2,6 dimethylundecane, 2,6,10 trimethyldodecane), two saturated monocyclic, (n-heptyl cyclohexane, n-octyl cyclohexane), two dicyclic (2 isopropyl decalin, 2,7 diisopropyl decalin), three polycyclic (perhydrophenanthrene, perhydropyrene, perhydrofluoranthene) naphthenic hydrocarbons and three cyclic hydrocarbons containing one monoaromatic ring (2 hexyl tetralin, 1-phenyl-3,3,5,5-tetramethylcyclohexane, dodecahydrotriphenylene) were investigated. All test substances are liquids at room temperature except dodecahydrotriphenylene. Additional information on CAS#s, visual depiction of structures, Log K_{ow}, predicted water solubility, purity and sources are provided in Tables S1 and S2. Slow-stir water solubility measurements have previously been reported for all test substances in this study except phenyl-tetramethylcyclohexane and dodecahydrotriphenylene (Letinksi et al. 2017). As part of this study, water solubility measurements were conducted for these two compounds following the same procedures previously described. Ten algal and six daphnid chronic limit studies were performed with some test involving common control treatments. All tests were performed following OECD Principles of Good Laboratory Practice (OECD, 1997). An overview of the toxicity studies conducted and corresponding test number identifiers are provided in Table S3 and described below.

Algal Tests

An algal culture was maintained in approximately 300 mL of nutrient media prepared with deionized water and reagent grade chemicals. Cell counts were performed weekly to ensure that the cells are in log phase of growth and to verify the identity and purity of the culture used as an inoculum in growth tests. A new culture was started weekly using inoculum from the previous culture. Cultures of *P. subcapitata* were held at 22 - 25°C under continuous illumination (8000 Lux ± 20%) provided by cool-white fluorescent bulbs. Algal toxicity tests were conducted in an environmental chamber with *P. subcapitata* in accordance with the OECD 201 (2011) test guideline. The initial density of algae inoculated was 1.0×10^4 cells/mL. All flasks were incubated at a temperature of 23°± 2°C under continuous lighting. Light intensity was measured using a LI-COR LI-250 meter and LI-210 photometric sensor. Temperature was monitored and pH was measured at start and end of each test. Cell density was determined for each test and control chamber using a hemacytometer and microscope. Cell density determinations were performed on three replicates at each observation interval. The growth rate in controls and treatments were determined from the regression equation of algal cell count with time time:

$$\mathrm{Ln}\,(N_{t,c}) = \alpha_c + \mu_c t \qquad (1)$$

where

$N_{t,c}$ = measured algal density at time t (cells/mL)

α_c = intercept term (not used in further estimation)

μ_c = growth rate (d^{-1})

t = exposure duration (d)

Statistical differences in growth rates between treatment and controls were determined by analysis of covariance (SAS, 2002). All test substances except the three saturated polycyclic hydrocarbons, tetramethylcyclohexane, and dodecahydrotriphenylene were dosed using the following strategy: (1) saturate initial test solutions using a "gas saturation" method and (2) maintain freely dissolved concentrations at saturation during the tests via a passive dosing method. A 5-10 ml volume of each neat liquid test substance was aerated using carbon scrubbed air at approximately 30 mL/minute in a "bubbler" apparatus and the saturated vapor was passed through glass tubing into a 2 L size graduated glass cylinder containing algal nutrient media that was pre-filtered through a sterile 0.45 µm filter, with 400 mg/L of $NaHCO_3$ added as a carbon source. The saturated vapor was then passed through a glass frit aerator near the bottom of the cylinder. The solution in the cylinder was also slowly stirred using a Teflon® coated stir bar and magnetic stirrer. This test system shown in Appendix S1 allowed the algal test media to be saturated with the hydrocarbon substances investigated in this study within a day.

The passive dosing device that was introduced into each algal test chamber was constructed of medical grade silicone tubing (0.3 mm internal diameter, 0.63 mm external diameter, 0.17 mm wall thickness, 20 cm in length) purchased from A-M Systems, Sequim, WA, USA. Silicone tubes were filled with approximately 15 µL of test substance and then used as the partitioning donor. First, the liquid test substance was pumped through the tube at a rate of 25 µL/min using a syringe pump. After 5 minutes of pumping, both ends of the tube were quickly tied together using a double knot to form a loop. This procedure was repeated to produce the required number of passive dosing devices for each treatment replicate. Upon inoculating 50 mL glass Erlenmeyer flasks with algae cells (see below), a loaded or control tube (no test substance) was immediately added. The flasks were then filled with test substance saturated or control (silicone tubing with no test substance) solution from the gas saturation system described above and sealed with no headspace using screw caps as illustrated in Appendix S1. Each chamber contained ~60 mL of test solution and Teflon stir bars. Three replicates were prepared for 24, 48 and 72 h algal cell measurements for the saturated and control treatments. Three additional flasks were filled with saturated test solution and a passive dosing device but with no algae. These flasks were also poisoned with a concentrated mercuric chloride solution to achieve a 50 mg/L concentration. These abiotic controls were included in the study design since differences in observed total concentrations between treatment and poisoned controls at the end of the test reflect the amount of test substance transferred from the passive dosing donor and accumulated by algae.

Due to the limited amounts of three saturated polycyclic hydrocarbon test substances available, the gas saturation method was not used to generate saturated media at test initiation. Instead, saturated batch solutions were prepared for treatment and controls by adding a passive dosing device containing the test substance or DI water (control) to algal nutrient media in approximately 4.5 L glass screw top aspirator bottles with Teflon® screw caps. The passive dosing device consisted of a 30 cm length of medical-grade silicone tubing (1.5 mm I.D., 2.0 mm O.D., 0.24 mm wall thickness) loaded with approximately 0.5 mL of test material for the treatment group or DI water for the control and then “tied off”. The loaded silicone tubing was carefully intertwined within the stir bar wing harness attached to a stir bar (~80 mm x 13 mm). The test solutions were then mixed on magnetic stir plates under ambient conditions for three days prior to the start of the toxicity study. Vortex height of each solution was 30% of the static solution height.

To maintain concentrations at saturation during tests an additional passive dosing device was added to each replicate test flask as previously described. This passive dosing device consisted of a 20 cm length of medical-grade silicone tubing (0.30 mm I.D., 0.64 mm O.D., 0.17 mm wall thickness) loaded with approximately 10 µL of test material for the treatment group or DI water for the control. Six replicates were prepared for control and treatment groups to allow algal density measurements at 24, 48, 72 and 96 h. Three replicates were also included as abiotic controls for test substance analysis at 72 and 96 h. This two-step procedure was also applied to conduct a second repeat test with trimethyldodecane to provide a basis for comparison with the gas saturation method described above.

Due to the unfavorable air-water partition coefficients for phenyl-tetramethylcyclohexane and dodecahydrotriphenylene (Table S1) vapor dosing was not applied. Instead two passive dosing approaches were piloted. For dosing the liquid, phenyl-tetramethylcyclohexane, 2 mL of neat test substance was added to a 12 mL clear glass vial with PTFE screw cap. Twelve red, commercially available silicone O-rings (O-ring West part # S70-M.75x10; ring thickness (ring cross-section) = 0.75 mm; inside diameter = 10 mm) were then added and allowed to equilibrate with the test liquid for 72 h as Illustrated in Appendix S1. Control O-rings were prepared in the same manner with methanol instead of test substance. All O-rings were rinsed at least three times in deionized water to remove test substance on the silicone surface of the loaded O-rings as well as any residual methanol from the dosed and control O-rings. Individual test chamber solutions for treatment groups and the control group were prepared by adding one rinsed silicone O-ring and a stir bar to a 50 mL Erlenmeyer flask containing 64 mL of algal media with no head space. All test chambers were sealed with PTFE screw caps and mixed for approximately 24 hours on magnetic stir plates before inoculation with algae. For dosing the solid, dodecahydrotriphenylene, 20 mg of test substance was added to 10 mL of silicone oil heated to 154 ºC followed by mixing using a glass stir bar on a heated magnetic stir plate. The silicone oil saturated with test substance was then loaded into a silicone tubing passive dosing device as described previously. Two controls were included with tubing loaded with and without clean silicone oil. All test chambers were sealed using PTFE screw caps and mixed for approximately 40 h on magnetic stir plates in the dark before initiating toxicity tests. Three replicates for treatment and control groups were prepared for algal density determinations at 24, 48 and 72 h. Abiotic controls were also included for chemical analysis at 72 h.

Test substance concentrations were measured immediately prior to study initiation in duplicate or triplicate and at study termination in at least triplicate. Samples characterizing initial exposure concentrations were taken from the "gas saturation" or silicone tubing dosing systems prior to adding the solution to the replicate test chambers. Samples at study termination were obtained from randomly sampling individual test replicates.

***Daphnia* Tests**

Eight to ten *Daphnia magna* Straus were cultured in 1-liter glass culture beakers with approximately 800 mL of reconstituted water. Cultures were started daily (at least five days per week) using eight to ten <24 hour old neonates from culture beakers between 12 and 18 days old, exhibiting ≤20% adult mortality. Cultures were transferred to fresh reconstituted water on regular intervals to ensure that ≤24 hour old neonates were available for studies and to start new cultures. Cultures of *Daphnia magna* Straus were fed *Pseudokirchneriella subcapitata* and supplemented with Vita-Chem or Microfeast PZ-20 suspension. The culture was fed daily or five days per week at a minimum. The algae was supplied by Aquatic Biosystems, Inc., Fort Collins, CO. Vita-Chem was supplied by Foster and Smith Aquatics, Rhinelander, Wisconsin while Microfeast PZ-20 was supplied by Salt Creek, Salt Lake City, Utah. Chronic 21 d limit toxicity tests followed the OECD (2012) test guideline. Ten replicates each containing one <24 h old neonate were used for all treatments. The test chambers were 125 mL size clear glass Erlenmeyer flasks containing approximately 140 mL of solution (no headspace). The test chambers were sealed with Teflon® lined screw lids. All tests were performed in moderately hard reconstituted water under a 16:8 h light/dark photo-period.

Observations for immobilization and neonate production were performed and recorded at approximately 24 h intervals after test initiation. After the appearance of the first brood, neonates were counted every other day. At the end of the test, the percent of adults surviving and the total number of living offspring produced per living parent at the end of the test was determined. Adult organisms were also measured (body length excluding the anal spine) at termination in order to assess potential effects on growth. To assess statistical significance of observed effects, a one-tailed t-test provided with the TOXSTAT software was used (WET, 1994).

For tests with 2,6 dimethyldecane and 2,6 dimethylundecane, a stock solution of test media containing food was prepared by adding 7 mLs of a 1.3 x 10^8 cells/mL suspension of *P. subcapitata* and 50 µL of VitaChem to provide 4.5 x 10^5 cells/mL, and 25 µL/L, respectively in dilution water. *V*itachem is a pre-stabilized, water soluble multi-vitamin supplement for finfish and aquatic invertebrate that contains natural lipids, fish oils and amino acids. This diet-containing media was then saturated using the gas saturation approach described for algal tests. However, custom made flow-through clear glass chambers, containing approximately 190 mL of solution (no headspace) were used. The top of the chamber contained two ports, an inlet which extended to the bottom of the chamber and an outlet, each of which contained Nitex screen, which prevented neonates from escaping through either port. Silicone tubing was used to connect the system, an Ismatec multi-head pump was used, with individual pump heads for each replicate, thus ensuring equal flow through each chamber. The saturated solution containing feed was pumped directly through the test chambers in a re-circulating system at a flow rate of 8.5 to 9.0 mL/minute. The test solution was then returned to the vapor dosing system in order to maintain test substance aqueous concentrations in equilibrium with the saturated bubbled air. This design provided three complete water volume exchanges of test chambers per hour and was found to overcome potential system losses (e.g. sorption to silicone tubing and test chambers) that might reduce water concentrations. The adults were removed from the test chamber to new test solutions on transfer days, when neonates were observed and counted. The test chambers were emptied into a culture dish in order to accurately count neonates. The adult was transferred back to its respective chamber which was then re-sealed and re-filled via the pump/re-circulating system. To characterize exposure concentrations during these tests, samples were collected for triplicate analysis at 16 time intervals over the course of the 21 d flow through test.

For the remaining tests, the two step process described for dosing algal test media was used. Test media was initially saturated with test substance by either the gas saturation method (n-octyl cyclohexane and a second, repeat test with 2,6 dimethylundecane) or by the passive dosing procedure with silicone tubing (saturated polycyclic compounds). A silicone tubing passive dosing device was included in test chambers to maintain test substance exposures. Test chambers consisted of 125 mL glass Erlenmeyer flasks that were completely filled with test solution with no headspace and sealed with Teflon® lined lids. One difference between this study design and the previously described tests was that the initial test media that was saturated with test substance did not contain food. Instead daphnids were fed daily by adding 0.5 mL of a 1.3×10^8 cells/mL suspension of *P. subcapitata* directly to test chambers to provide 4.2 to 6.2×10^5 cells/mL. Test organisms were also fed daily with 0.05 mL of Microfeast PZ-20 suspension. Microfeast is microalgae dietary supplement that is used to support healthy early stage growth in crustaceans.

Dosed media were prepared and renewed at 48-hour (±2 hour) intervals. Renewals were performed by transferring each parent daphnid, via glass pipette, and the passive dosing device to freshly dosed solutions. A minimum of eight water samples were taken to characterize test substance exposures in “new” solutions at the start of renewals. Individual test chambers were then sampled in triplicate or quadruplicate on a minimum of eight occasions to characterize exposure concentrations in “old” solutions at the end of renewals. Temperature, pH and dissolved oxygen concentrations were monitored daily.

***Ceriodaphnia* Tests**

Ceriodaphnia dubia were maintained in 20 mL glass scintillation vials filled with moderately hard reconstituted water supplemented with Na_2SeO_4 at 2µg/L Se and 1µg/L Vitamin B12 at 25 ± 2°C. Stock cultures were transferred to fresh reconstituted water daily and fed a suspension *P. subcapitata* and yeast-cereal leaves-trout mixture (YCT). Stock cultures of test organisms were started at least three weeks before the brood animals were needed. Chronic limit toxicity studies were based on the static-renewal standard test guideline (USEPA, 2002). Ten replicates each containing one <24 h old neonate were used for control and treatment groups. Test chambers were 20 mL glass scintillation vials containing one *C. dubia* and approximately 22 mL of test solution with no headspace. Each chamber was closed with PTFE-lined screw caps. All tests were performed in moderately hard reconstituted water under a 16:8 h light/dark photo-period.

Observations for immobilization were performed and recorded at 24 ± 1 hour intervals. The adults were transferred via pipette to chambers containing fresh test solution daily. Neonate presence and enumeration from each adult was performed following the adult transfer on a daily basis beginning on the third day of the test. To allow production of three broods per test guideline requirements the duration of tests were 6-7 days. Chronic endpoints were calculated using cumulative reproduction data over the duration of the test. After for checking for normality, analysis of variance was used to determine if reproduction in dosed animals were statistically reduced relative to the control group using JMP v. 13 (JMP, 2016).

For dosing phenyl-teramethylcyclohexane and dodecahydrotriphenylene, the same approach described for algal tests was followed. Phenyl-teramethylcyclohexane involved equilibrating 5 silicone O-rings (O-ring

West part # S70-M.75x10; ring thickness (ring cross-section) = 0.75 mm; inside diameter = 10 mm) for 72 h in the neat test liquid. Dosed media was then prepared by adding 5 rinsed silicone O-rings to 0.5 L moderately hard reconstituted water with micronutrients and on a stir plate for 24±1 hour. Control and treatment group media were prepared daily using the initially dosed O-rings. A saturated methanol stock solution of Dodecahydrotriphenylene was prepared. One mL of this stock was then loaded into 80 cm of silicone tubing (AM-systems, catalog# 807600, 1.5mm ID, 2.0mm OD). The loaded tubing was tied to a Teflon® coated stir bar and added to 4 L of dilution water in a 4 L aspirator bottle on a magnetic stir plate for 46 h prior to use in toxicity tests.

In all tests, daily renewals consisted of treatment solution and control solution being distributed into new test vials and the *C. dubia* being relocated from the "old" treatment vials to the "new" treatment vial. Upon each daily renewal each replicate was fed the appropriate volume of feed. Duplicate water samples were taken from each treatment solution and the control on day 0 and 5 representing "new" solutions and on day 1 and 6 representing "old" solutions for test substance analysis. "Old" samples were composites of treatment replicates to provide sufficient volume for extraction. Temperature, pH and dissolved oxygen concentrations were monitored daily.

Test Substance and Water Quality Analysis

Test substance specific analytical methods were developed, validated and applied to document measured exposures in all tests. Methods were developed to quantify total concentrations in test media and tailored to provide the required sensitivity needed to reliably characterize exposures of the poorly water soluble substances investigated. The more volatile test substances were measured using headspace SPME-GC-MS or headspace Trap-GC-MS. The less volatile compounds were analyzed using direct immersion SPME-GC-MS. Standards were prepared by spiking microliter amounts of the individual test compounds diluted in acetone into the same blend water used to prepare the respective algae or daphnia media. The standard concentrations in water spanned the calibration ranges and each contained a constant concentration of the selected internal standard as detailed in Table S4. Samples of 10 to 20 mLs for test substance confirmation were collected and similarly processed as the standards with the same concentration of the internal standard added to each prior to SPME or headspace extraction. The incorporation of internal standards reflects best practice when performing partition-based analytical extractions. The MS detector was operated in the selective ion monitoring mode in all methods. Further details on the specific equipment used, along with information on internal and calibration standards and detection limits are provided in Table S4. Water quality monitoring of exposure solutions was performed for all toxicity tests as stipulated in the previously cited OECD test guidelines.

RESULTS & DISCUSSION

Algal Tests

Light intensity, temperature and initial and final pH measurements for all tests are summarized in Table S5. An increase of the final pH was observed for both controls and treatments even though the algal medium had been enriched with additional NaHCO3 to reduce the pH increase. A pH increase is often unavoidable in a closed no-headspace test system under the standardized test guideline conditions that specify a required initial algal density, growth rate and test duration (Mayer et al, 2000). The final pH ranged from 8.3 to 9.2, which had no discernable effects on the algal growth over the test. Algal growth rates are summarized in Table S6 and shown in Figure 1. The growth rate over 72 h in controls across the ten limit tests averaged 1.34 d^{-1} (range 1.08 to 1.70). These tests were considered to meet the test guideline requirement that requires cell density in the control increase by ≥16 fold within 72 h (OECD, 2011). In addition, the coefficient of variation (CV) for average specific growth during the 72-hour period in control replicates did not exceed the 7% requirement in 8 out of the 10 tests. For tests 1 and 2 reported in

Figure 1 the CV for the specific growth rate in controls slightly exceeded this criterion with values of 7.5 and 8.7%, respectively (Table S6). The CV for section by section (i.e., day to day) specific growth rates in the control replicates met the guideline criterion of 35% for all experiments except test 1 (CV=39%). This higher variability was due to an initial slower growth rate during the first day of this experiment. However, this deviation does not appear to impact test interpretation since both test substances included in this test (pentamethylheptane, heptylcyclohexane) were shown to cause a statistically significant effect of growth rate when compared to the control along with three of the other more water soluble hydrocarbons (dimethyldecane, phenyltetramethycylohexane and 2-hexyl tetralin) (Figure 2). While none of polynaphthenic compounds inhibited growth after 72 h, perhydrophenanthene caused a slight (5%) but statistically significant effect on growth after 96 h. However, exposure to the two less soluble compounds from this class (perhydropyrene and perhydrofluoranthene) showed no effects on growth over 96 h (Table S7).

Table 1 summarizes measured exposures at the beginning and end of algal limit tests. The vapor dosing method (VPDT) yielded initial exposure concentrations that were near or above measured water solubility values for all compounds except 2-hexyltetralin. This substance exhibited the lowest air-water partition coefficient of the compounds tested (Table S1) and thus appears insufficiently volatile to enable saturation of the aqueous test media using the vapor dosing system employed in this study. This learning led to abandoning the use of vapor dosing for the two remaining two monoaromatic naphthenic substances in subsequent tests. In contrast, the initial concentration of trimethyldodecane delivered via vapor dosing was almost two orders of magnitude higher than the reported solubility and likely reflects neat liquid aerosols in the saturated vapor that were transferred via gas bubbles to the aqueous test media. Initial concentrations for test substances dosed via passive dosing with neat substance (PDT) or saturated silicone oil (PDTSO) loaded into silicone tubing or O-rings (PDOR) yielded measured exposures that were within a factor of two of water solubility measurements (Table 1). Analytical results obtained for poisoned controls at the end of limit tests showed that concentrations were similar or increased relative to initial concentrations (Table S8). Increases were most pronounced for 2 hexyl tetralin which exhibited initial concentrations well below solubility. These results confirm the effectiveness of the passive dosing device applied for achieving saturation. In the case of the test with trimethyldodecane using vapor dosing, concentrations in poisoned controls dropped slightly but remained at a mean concentration that was a factor of 70 above water solubility again suggesting the presence of neat test substance. For several other test compounds, concentrations in poisoned controls were maintained at or up above the solubility limit (Table S8). The higher than expected concentrations may be in part explained by the fact that the reported solubilities in Table 1 were generated at 20°C while the algal tests were performed at about 24°C (Table S5). It is also possible that traces of dissolved organic carbon in algal test media may have contributed to an apparent solubility enhancement particularly for the more hydrophobic substances.

We expected that total concentrations of the investigated hydrocarbons would increase at test termination due to the elevated biomass that enhances the capacity of the aqueous media for hydrophobic organic compounds (Birch et al, 2012). While concentrations generally increased from the start to end of tests, the magnitude of the observed increase differed widely across test substances (Table S8). As detailed in Appendix S2, differences in observed concentrations in treatment and poisoned controls were used to estimate concentrations in algae at test termination. These data were compared to predictions derived from an algal bioconcentration model and used to further explore internal algal residue-effect relationships. Insights obtained from this analysis were inconclusive and highlighted the need for further kinetic studies, including consideration of the potential role of test substance biodegradation and/or algal biotransformation, for elucidating the underlying mechanisms that can limit the accumulation and preclude growth inhibition despite hydrocarbon exposures at aqueous solubility.

Daphnid Tests

Water quality data summarized in Table S9 was found acceptable across all tests. No control mortality (i.e. immobilization) was observed in any of the six chronic limit tests. Neonate production in 21 d *Daphnia* and three brood *Ceriodaphnia* tests met guideline requirements and ranged from 91 to 184 and 29 to 30, respectively (Table S10). No effects on adult survival were observed for all hydrocarbon tested with the exception of perhydrophenanthrene and phenyl-tetramethylcyclohexane. For perhydrophenanthrene, three out of the ten adults were immobilized within the first three days of the test. In contrast, complete mortality was observed in the limit study for phenyl-tetramethylcyclohexane within 48 h. As a result no neonate production was observed at the limit concentration tested since all adults died. Neonate production in the four *D. magna* and two *C. dubia* limit tests are reported in Table S10 and illustrated in Figures 2 and 3. Results show that none of the limit tests with the other hydrocarbons tested caused significant differences in reproduction when compared to the controls. Adult length of D. magna at the end of the test was also included as an endpoint to assess potential effects on growth. No effects on length were observed except in one of the two limit tests with dimethyldecane (Table S10). However, while the difference in adult length was statistically significant in this one study, this effect represented only a 2% change and is not judged biologically significant. Application of the target lipid model to *D. magna* and *C. dubia* chronic toxicity data sets that are available for more water soluble hydrocarbons indicates these species exhibit very similar sensitivities as evidenced by reported critical target lipid body burdens of 4.1±1.3 and 3.7±0.8 μmol/$g_{octanol}$, respectively (McGrath et al. 2018). Thus, given the expected comparable sensitivity to *D. magna* coupled with the shorter duration and cost effectiveness of the *C. dubia* guideline, this alternative chronic test appears to be a logical choice for elucidating chronic toxicity cut-offs.

Table 2 summarizes the results of analytical confirmation of limit test concentrations at the beginning and end of test renewals. The geometric mean measured concentration was within a factor of two of the reported solubility for all test substances except phenyltetramethylcyclohexane which was about a factor of three lower but still sufficiently elevated to cause obvious toxic effects.

Comparison to Literature Data

Limited toxicity data are available on branched alkanes and naphthenic hydrocarbons to compare directly to the data from this study. The toxicity of pentamethylheptane to *P. subcapitata* was investigated in a limit study using a water accommodated fraction (WAF) dosing approach at a nominal loading of 1000 mg/L using a static test. No effects were observed and the 72-hr EL_{50} for growth rate was reported as >1000 mg/L (ECHA, 2018a). While this study was judged reliable, no analytical confirmation of exposure concentrations was performed. In another study on a similar compound, isododecene, no effects were observed in a 21 d OECD 2011 *D. magna* guideline study where a 21 d NOEC >16 µg/L was reported based on measured test concentrations (BASF AG, 2004).

Toxicity studies using standard test guidelines for algae growth and *D. magna* reproduction tests have also been reported for 2,6,10 trimethyldodecane (ECHA 2018b). In these tests solvent was used to increase apparent solubility of this test substance at test start up to measured concentrations of 86 µg/L. No effects were observed on *P. subcapitata* at the highest exposure concentration and the reported 96 h NOEC based on the geometric mean measured concentration was > 9.3 µg/L. For evaluating chronic effects to *D. magna,* organisms were exposed to mean measured concentrations of 12 to 77 µg/L under flow-through conditions for 21 days. There were no statistically significant treatment-related effects on survival or dry weight at concentrations ≤77 µg/L. Daphnids exposed at 77 µg/L had statistically significant reductions in length and reproduction in comparison to the control with a reported NOEC of 54 µg/L. However, the reliability of adopting these results are low given the NOEC is more than a hundred fold greater than the measured solubility limit (Table 1). Consequently, results do not reflect the intrinsic substance hazard but rather the likely confounding influence of physical effects of undissolved test substance liquid on the test animals.

Chronic toxicity tests based on measured concentrations for n-undecane have been reported with a 72 h algal growth and 21 d Daphnia magna NOEC of 5.7 and 5.9 µg/L, respectively (Ministry of the Environment Japan, 2018). For comparison, the measured slow-stir water solubility of this test substance is 14 µg/L (Letinski et al. 2016). In an earlier unpublished study performed in our lab using a gas saturation system to enable vapor dosing of test media analogous to that used in this study, 21 d *D. magna* static renewal tests were performed for C10-C12, isoalkanes, < 2% aromatics and C11-C12, isoalkanes, < 2% aromatics (ExxonMobil Biomedical Sciences, Inc., 2005). The measured solubility of these two test substances based on vapor dosing was 79±2 and 36±2 µg/L, respectively. Both test substances were shown to cause chronic effects with reported NOECs based on measured geometric mean concentrations of 25 and 11 µg/L. These data imply a chemical activity based chronic effect threshold for C10-C12 alkanes of 0.3 to 0.4. Trac et al. (2019) have applied a novel closed vial headspace dosing method to investigate the toxicity of n-nonane, n-undecane, isodecane and n-tridecane to algae and springtails. For nonane, a 72 h EA50 for algal growth inhibiton of 0.4 (0.25-0.35) and a 7 d LC50 for springtail survival of 0.3 (0.25-0.35) was reported. Based on the reported activity-effect relationships, EA10 values were approximately a factor of two lower. Effects were also observed for the other alkanes investigated but results were not expressed in terms of chemical activity to allow further comparison.

Several toxicity studies are available on hydrocarbon solvents using the WAF dosing method based on nominal substance loading. No effects on algal growth rates were reported for C10-13 isoalkanes, C10-

C12 isoalkanes, <2% aromatics, and C11-C14, n-alkanes, isoalkanes, cyclics, <2% aromatics with 72 h NOELs of > 1000, >100 and >1000 mg/L, respectively (ECHA 2018c,d,e). Similarly, no effects have been reported in 21 d *D. magna* chronic studies at the highest loading investigated (ECHA 2018e). For C13-C16, isoalkanes, cyclics, <2% aromatics, C13-C16, isoalkanes, cyclics, <2% aromatics a NOEL of > 5 mg/L was reported. For C13-C18, n-alkanes, isoalkanes, cyclics, <2% aromatics, C14-C17, n-alkanes, <2% aromatics, C14-C18, n-alkanes, isoalkanes, cyclics, <2% aromatics, C14-C20, n-alkanes, <2% aromatics and 16-C20, n-alkanes, isoalkanes, cyclics, <2% aromatics, 21 d NOELs of > 1000 mg/L were observed. Two studies with *C. Dubia* have also been conducted for C12-C15, n-alkanes, <2% aromatics and C14-C17, n-alkanes,<2% aromatics (ECHAS 2018e). Based on mortality and reproduction, a NOEL > 100% WAF was reported. However, the loading of test substance used was not specified. In a recent study by Whale et al (2018), algal toxicity data were compiled for various aliphatic solvents produced from catalytic processing using natural gas as the feedstock. Multiple 72 h NOELs > 100 mg/L for *P. subcapitata* for a range of substances from C8–C11, n-alkanes, isoalkanes, <2% aromatics to C18–C24, isoalkanes, <2% aromatics were reported. This study also reported a 21 d chronic NOEC for D. magna of > 100 mg/L for a solvent defined as C9–C11, n-alkanes, isoalkanes, <2% aromatics. While WAF studies discussed above provide a convenient and standardized method to evaluate the comparative hazard of poorly water soluble substances, given the multi-constituent nature and dissolution behavior of these substances and lack of exposure characterization it is not possible to use these data to better define solubility cut-offs. In contrast, available literature studies for alkanes for which measured concentrations are reported appear to be consistent with results from this study.

Based on the chronic critical target lipid body burdens (CTLBB) for algal of 10.7±2.9 $\mu mol/g_{octanol}$ versus *D. magna* and *C. Dubia* test endpoints that are deduced from fitting empirical chronic hydrocarbon toxicity using the target lipid model (McGrath et al. 2018) as previously discussed, we had hypothesized the daphnid endpoints would be more sensitive. While data are limited, it does not appear that the 21 d test for *D. magna* is in fact more sensitive than the algal growth endpoint (c.f. Tables 1 and 2). This difference in sensitivity may be attributed to organism size and the faster toxicokinetics associated with algal tests (Kwon et al. 2016). In contrast for phenyl-tetramethylcyclohexane, which was the most water soluble compound tested, the *C. Dubia* chronic test was indeed more sensitive than algal growth with a reported 6 d EC_{10} of 13 μg/L when compared to the 72 h algal EC_{10} of 67 μg/L. This difference in sensitivity is in better agreement with the relative sensitivity inferred from lower estimated chronic CTLBB for the *C. Dubia* endpoint. This indicates that differences in toxicokinetics between algae and daphnids appear less important for more soluble test substances.

A number of recent algal toxicity studies with hydrophobic compounds, including parent and alkyl polyaromatic hydrocarbons, when combined with data from this study can be used to further investigate empirical toxicity cut-offs (Table 3). Several important insights can be gleaned from this compilation. First, results from the study reported by Kang et al. (2016), which relied on solvent spiking, found that a number of the more water soluble compounds tested, such as dimethylfluorene, dimethyl phenanthrene and dimethylanthracene, did not exhibit toxicity at concentrations approaching the solubility limit. In contrast, all the remaining studies, which relied on passive dosing methods, demonstrated effects for test substances with a corresponding water solubility above 5 μg/L. This consistency across studies highlights the advantage of applying passive dosing for reliable aquatic hazard characterization of hydrophobic compounds. Second, for test compounds with water solubilities below 5 μg/L, growth effects on algae are not observed at the solubility limit of the test substance. Third, the octanol-water partition coefficient appears to be a much less effective test substance property for delineating toxicity cut-offs than the water solubility limit consistent with the conclusions reported by Stibany et al. (2020). For example, dodecylbenzene which has a predicted Log Kow value of 7.94 was shown to exhibit algal toxicity while chrysene with a calculated Log Kow value of 5.52 was found to be not toxic at exposure concentrations corresponding to the solubility limit.

The new experimental data generated in this study significantly expands current understanding of the effects of non-polyaromatic hydrocarbons using standard aquatic chronic toxicity tests. This information supports aquatic hazard classification of substances under the globally harmonized system for hazard classification and labeling of chemicals as well as toxicity evaluations of hydrocarbons that are included in various regulatory schemes including PBT assessments. These data also can be used to support validation and refinement of computational models used in hazard and risk assessments of petroleum substances that include the different hydrocarbon classes investigated in the present work (Salvito et al. 2020).

A potential disadvantage of o-ring passive dosing used in this study is that hydrophobic solids may have limited solubility in methanol, the typical loading solvent for the o-ring dosing technique. This challenge is especially significant when attempting to perform tests at maximum water solubility. An advantage of using the tubing approach is that hydrocarbon solids have greater solubility in silicone oil than methanol and the crystals that do not dissolve in silicone oil are retained when loaded into the silicone tubing. However, a more systematic evaluation of the advantages and disadvantages of both methods were beyond the scope of this study. Further work is needed to systematically assess the advantages and limitations of the various passive dosing approaches presented in this work.

Summary

Vapor and passive dosing methods were applied to evaluate chronic effects for a range of poorly water soluble hydrocarbons with supporting analytical confirmation of actual test exposures using algal and daphnid toxicity limit tests. Results indicate a solubility cut-off for chronic toxicity of structures containing 13-14 carbons for branched and cyclic alkanes and 16-18 carbons for monoaromatic naphthenic hydrocarbons. This work highlights the advantages of linking several passive dosing methods to chronic limit tests for hydrophobic test substances. A key finding is that water solubility appears to provide a useful parameter for defining toxicity cut-offs. Based on the compounds investigated in this study coupled with available literature data in which passive dosing was used, substances with a measured water solubility below 5 µg/L did not exhibit effects in the chronic toxicity assays investigated. However, caution should be exercised in extrapolating this rule of thumb to other compound classes. Further work is needed to systematically evaluate the advantages and disadvantages of using silicone tubing versus o-rings as a passive dosing format for solids and liquids. The methods described in this study should be broadly applicable to address this challenge for both hydrophobic organic liquid and solid substances. Additional research is needed for applying such passive dosing test designs to further assess if empirical water solubility-based toxicity cut-offs can be established for other compound classes.

The application of these novel dosing approaches to degradable substances raises new questions about the potential contributing role that transient metabolites formed during toxicity test exposures might play in complicating hazard interpretation. Further information on the bioconcentration kinetics and quantitative importance of microbial and biotransformation on substance uptake during chronic toxicity tests is also needed to better understand the mechanistic basis explaining observed toxicity or lack of effects. It is recommended that in future passive dosing algal limit studies with hydrophobic substances, dissolved and total concentrations in test media as well as in algal biomass and dissolved organic carbon are collected so that preliminary toxicokinetic model framework detailed in the supplemental information can be better calibrated and tested for toxicity prediction.

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Table 1 Algal Toxicity Limit Test Exposures and Inhibitory Effects on Growth Rate

Test Substance	Dosing Method	Slow-Stir Water Solubility (µg/L)	Initial Exposure Concentration (µg/L)	Final Exposure Concentration (µg/L)	Geometric Mean Concentration (µg/L)	% Algal Growth Inhibition
branched alkanes						
2,2,4,6,6-pentamethylheptane	VPDT	23.0 (4.2)	28.0 (3.6)	263.8 (24.2)	85.9	19
2,6-dimethyldecane	VPDT	11.0 (3.5)	11.5 (4.3)	26.3 (15.6)	17.4	37
2,6-dimethylundecane	VPDT	2.7 (2.8)	7.6 (11.3)	24.5 (13.6)	13.6	NS
2,6,10-trimethyldodecane	VPDT	0.3 (2.1)	31.3 (10.6)	14.7 (10.2)	21.5	NS
2,6,10-trimethyldodecane	PDT	0.3 (2.1)	0.5 (2.2)	2.4 (14.1)	1.1	NS
mononaphthenics						
n-heptylcyclohexane	VPDT	6.2 (5.7)	4.0 (13.9)	124.2 (16.6)	22.3	23
n-octylcyclohexane	VPDT	1.4 (2.8)	2.8 (0.0)	15.8 (10.1)	6.7	NS
dinaphthenics						
2-isopropyldecalin	VPDT	25.0 (6.4)	19.0 (10.5)	115.7 (29.7)	46.9	NS
2,7-diisopropyl decalin	VPDT	1.8 (6.3)	0.8 (4.3)	8.3 (42.2)	2.6	NS
polynaphthenics						
perhydrophenanthrene	PDT	20.0 (1.3)	30.6 (5.7)	21.1 (12.8)	25.4	5*
perhydropyrene	PDT	4.7 (0.7)	3.1 (13.2)	16.6 (5.5)	7.2	NS
perhydrofluoranthene	PDT	3.7 (2.0)	6.1 (0.6)	14.1 (57.7)	9.3	NS
monoaromatic naphthenics						
1-phenyl-3,3,5,5-tetramethylcyclohexane	PDOR	66.5 (18)	104.1 (10.4)	285.4 (22.3)	172.4	33**
2-hexyltetralin	VPDT	15.0 (5.8)	1.2 (4.3)	514.7 (5.3)	24.9	82
dodecahydrotriphenylene	PDTSO	2.8 (11)	3.0 (11.6)	3.9 (16.1)	3.4	NS

( ) = coefficient of variation calculated as standard error divided by mean x 100%
VPDT = initial vapor phase dosing followed by passive dosing of neat test liquid in silicone tubing
PDT = passive dosing of neat test liquid in silicone tubing
PDOR = passive dosing of neat test liquid loaded into silicone O-rings
PDTSO = passive dosing of test substance saturated silicone oil loaded into tubing silicone
NS= growth rate not significantly different from control (p=0.05)
*a statistically significant 5% reduction in growth rate was observed after 96 h exposure but not after 72 h
**results of definitive testing indicate the 72 h EC_{10} = 67 µg/L for algal growth rate with 95% confidence limits of 57-74 µg/L

Table 2 Summary of Invertebrate Toxicity Test Exposures and Chronic Effects

Test Substance	Dosing Method	Slow-Stir Water Solubility (µg/L)	New Exposure Concentration (µg/L)	Old Exposure Concentration (µg/L)	Geometric Mean Concentration (µg/L)	Adverse Effect?
branched alkanes						
2,6 dimethyl decane	VDFT	11.0	NA	NA	10.1 (2.8)	No
2,6 dimethyl undecane	VDSR	2.7	1.4 (11.7)	4.0 (22.9)	2.4	No*
2,6 dimethyl undecane	VDFT	2.7	NA	NA	2.3 (10.6)	No
mononanpthenics						
n-octyl cyclohexane	VDSR	1.4	4.7 (21.8)	1.6 (22.3)	2.7	No
polynaphthenics						
perhydrophenanthrene	PDTSR	20.0	26.2 (21.7)	6.0 (12.3)	12.5	Yes**
perhydropyrene	PDTSR	4.7	3.0 (13.9)	1.3 (17.2)	2.0	No
perhydrofluoranthene	PDTSR	3.7	3.8 (9.3)	0.9 (10.2)	1.8	No
monoaromatic naphthenics						
1-phenyl-3,3,5,5-tetramethylcyclohexane	PDORSR	66.5	42.4 (10.4)	13.1 (22.3)	23.6	Yes***
dodecahydrotriphenylene	PDTSOSR	2.8	2.0 (5.2)	2.1 (20.2)	2.0	No

( ) = coefficient of variation calculated as standard error divided by mean x 100%
VDFT = vapor dosing of test media using flow-through exposure
VDSR = vapor dosing using static renewal exposure
PDTSR = passive dosing of test media with neat test substance in silicone tubing using static renewal exposure
PDORSR = passive dosing of test media with neat test substance loaded into O-rings using static renewal exposure
PDTSOSR = passive dosing of test media with test substance saturated silicone oil loaded into tubing using static renewal exposure
NA= not applicable as flow through test in which samples collected for substance analysis over course of 21 d test
*the statistically significant 2% reduction in body length is not judged as likely biologically significant
** 30% adult mortality was observed within 72 h; however, no chronic effects were observed for reproduction or growth endpoints
***results of definitive chronic testing indicate the 6 d EC_{10} = 13 µg/L for neonate reproduction with 95% confidence limits of 9-18 µg/L.

Table 3. Summary of Algal Growth Inhibition Studies

Test Substance	Mol. Wt. (g/mol)	Log[1] K_{ow}	Water Solubility[2] (µg/L)	Algal Growth Endpoint	Measured Effect Concentration (µg/L)	Citation
bromochlorophene	426.9	6.12	8400	72 h EC10	50	E
n-nonane	128.3	5.34	253	72 h EC10	50.6*	F
9,9-dimethylfluorene	194.3	4.66	860	48 h EC50	NT**	C
1-methyl pyrene	216.3	5.48	100	48 h EC50	82	C
1-methyl pyrene	216.3	5.48	100	72 h EC10	72	B
1-phenyl-3,3,5,5-tetramethylcyclohexane	216.4	6.55	254	72 h EC10	67	A
3,6-dimethylphenanthrene	202.3	5.54	37	48 h EC50	NT**	C
2-isopropyldecalin	180.3	5.52	25	72 h EC10	46.9	A
2,2,4,6,6-pentamethylheptane	170.3	5.81	23	72 h EC19	85.9	A
perhydrophenanthrene	192.3	5.22	20	96 h EC5	25.4	A
2-hexyltetralin	216.4	6.83	15	72 h EC82	24.9	A
dodecylbenzene	246.4	7.94	12	72 h EC10	12	D
2,6-dimethyldecane	170.3	6.09	11	72 h EC37	17.4	A
9,10 dimethylanthracene	206.3	5.44	7.9	48 h EC50	NT**	C
n-heptylcyclohexane	182.3	6.54	6.2	72 h EC10	22.3	A
perhydropyrene	218.4	5.94	4.7	96 h EC10	>7.2	A
perhydrofluoranthene	218.4	5.94	3.7	96 h EC10	>9.3	A
dodecahydrotriphenylene	240.4	7.89	2.8	72 h EC10	>3.4	A
2,6-dimethylundecane	170.3	6.09	2.7	72 h EC10	>13.6	A
7-methylbenz[a]anthracene	242.3	6.07	2.7	48 h EC50	NT**	C
dibenzo[a,h]anthracene	278.4	6.7	2.5	72 h EC10	>0.15	B
2,7-diisopropyl decalin	222.4	6.85	1.8	72 h EC10	>2.6	A
7,12-dimethylbenz[a,h]anthracene	256.4	6.62	1.8	48 h EC50	NT**	C
benzo[a]pyrene	252.3	6.11	1.5	72 h EC10	>0.9	B
n-octylcyclohexane	196.4	7.03	1.4	72 h EC10	>6.7	A
chrysene	228.3	5.52	0.7	72 h EC10	>3.4	B
2,6,10-trimethyldodecane	212.4	7.49	0.3	72 h EC10	>21.5	A
2,6,10-trimethyldodecane	212.4	7.49	0.3	72 h EC10	>1.1	A
benzo[ghi]perylene	276.3	6.70	0.14	72 h EC10	>0.28	B

A=This study; B= Bragin et al. 2016; C=Kang et al. 2016; D=Stibany et al. 2017a; E=Stibany et al. 2017b; F=Trac et al. 2019; NT= growth inhibition was not observed at nominal concentrations spiked slightly below the water solubility limit; measured exposures were not verified

[1] Predicted using KOWWIN v1.68 in EPISuite v4.1

[2] Measured solubilities obtained from Letinksi et al. 2016; Kang et al. 2017; Stibany et al. 2017 a,b

*Determined by multiplying estimated EA10 by the water solubility value reported by Letinski et al. 2016

** EC50s could not be determined =using passive dosing but measured exposure concentrations were not reported

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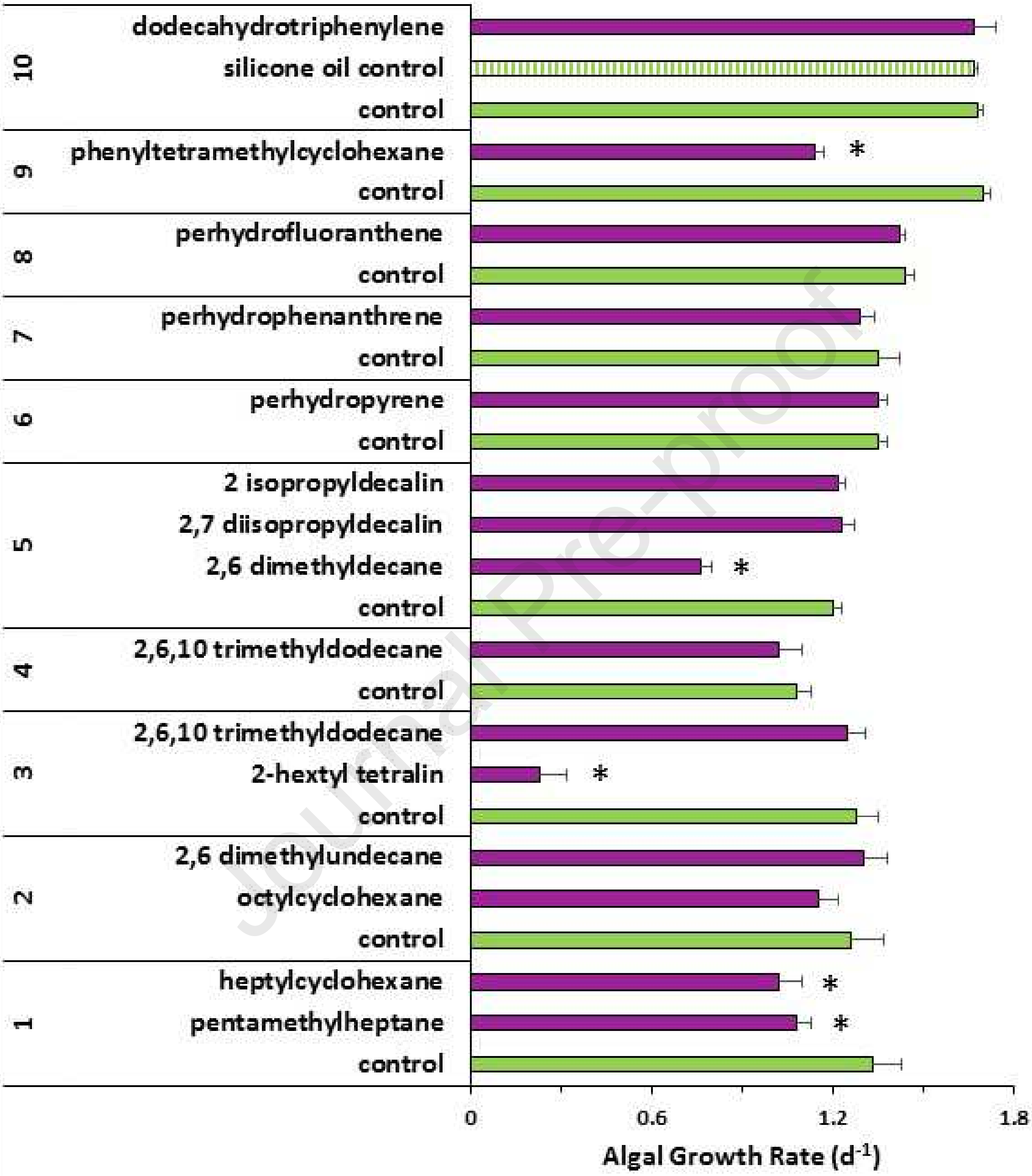

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814 Figure 1. Algal growth rates in control (green bars) and hydrocarbon dosed treatments (purple bars);
815 Asterisks indicate growth rates are statistically different (p<0.05) from corresponding controls. The
816 numbers to the left of the figure denote the test number as described in Table S3.
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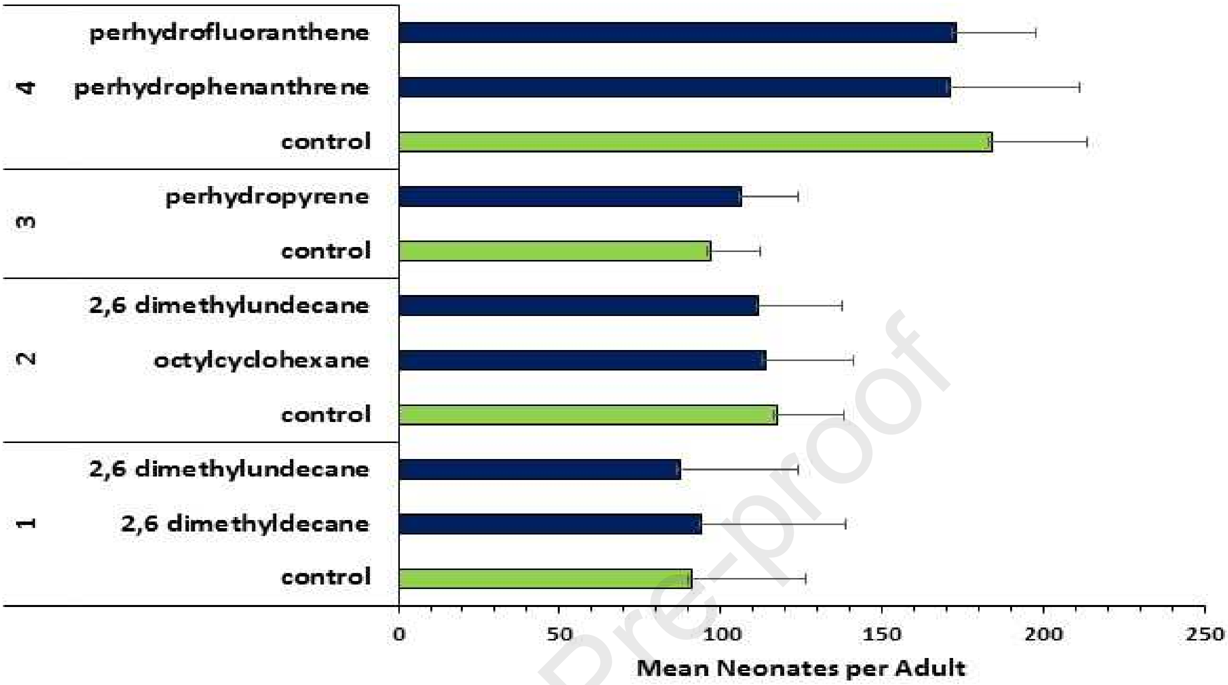

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820 Figure 2. *Daphnia magna* mean neonate production per adult in control (green bars) and hydrocarbon
821 dosed treatments (blue bars); Observed reproduction in hydrocarbon test exposures was not statistically
822 different ($p<0.05$) from corresponding controls. The numbers to the left of the figure denote the test
823 number as described in Table S3.

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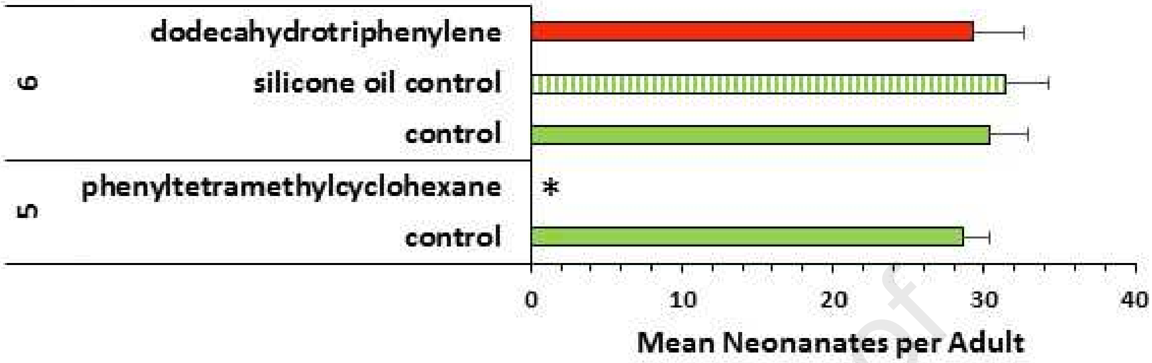

Figure 3. *Ceriodaphnia dubia* mean neonate production per adult in control (green bars) and hydrocarbon dosed treatments (red bar); Asterisk denotes no data since complete adult mortality was observed precluding reproduction. The numbers to the left of the figure denote the test number as described in Table S3.

HIGHLIGHTS:

- Novel dosing methods used to evaluate chronic toxicity at water solubility limit
- Measured concentrations confirmed exposures maintained over test duration
- Data used to establish empirical chronic toxicity cut-offs for hydrocarbons
- Aqueous solubility serves as a useful property for delineating toxicity cut-offs

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: