Marine Pollution Bulletin 165 (2021) 112151

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Miniaturised marine tests as indicators of aromatic hydrocarbon toxicity: Potential applicability to oil spill assessment

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ARTICLE INFO

Keywords:
Toxicity kits
Rapid assessment
Aromatic hydrocarbons
Passive dosing
Oil spill
Target lipid model

ABSTRACT

Assessing oil spill toxicity in real time is challenging due to dynamic field exposures and lack of simple, rapid, and sensitive tests. We investigated the relative sensitivity of two commercially available marine toxicity tests to aromatic hydrocarbons using the target lipid model (TLM). State of the art passive dosing in sealed vials was used to assess the sensitivity of brine shrimp (*Artemia franciscana*) and rotifer (*Brachionus plicatilis*). Organisms were exposed to toluene, 1-methylnaphthalene and phenanthrene for 24 h. Toxicity results were analysed using the TLM to estimate the critical target lipid body burden and support comparison to empirical data for 79 other aquatic organisms. Our findings demonstrate the applicability of passive dosing to test small volumes and indicate that the two rapid cyst-based assays are insensitive in detecting hydrocarbon exposures compared to other aquatic species. Our results highlight the limitations of applying these tests for oil pollution monitoring and decision-making.

1. Introduction

Crude oil is a complex mixture of many different compounds including, predominantly, saturated hydrocarbons and aromatic hydrocarbons, with lower amounts of other structures which may contain sulphur, nitrogen, oxygen or trace metals (Beens and Brinkman, 2000). The specific composition of the crude oil can have a strong impact on fate and effects on aquatic organisms following release into the environment. This is because the properties that control fate and toxicity vary widely with the size and nature of the hydrocarbons in the crude oils. These properties are particularly challenging when responding to accidental spills; where the exposures are variable due to physical forces (e.g., wind and waves) and also the variable and changing composition of the oil itself. These processes further confound identification, and interpretation of potential effects (Colvin et al., 2020).

Hydrocarbon toxicity varies with the structure of the molecule, which in turn varies with the composition of the oil. Volatile organic chemicals such as benzene, toluene, ethylbenzene, xylenes (BTEX) and polycyclic aromatic hydrocarbons (PAHs) can interfere with survival, membrane permeability and enzyme function which may lead to toxic, mutagenic, carcinogenic or teratogenic effects (Claxton et al., 1991; Incardona et al., 2014; Varjani et al., 2017). These properties have resulted in the listing of many PAHs as priority substances by both the EU and US EPA (European Union, 2013; US EPA, 2015).

Biomonitoring can be used in the event of a pollution incident as a tool to assess or predict biological impacts associated with variable oil exposure and composition during a spill (Wieczerzak et al., 2016). In the case of oil spill response it may also be possible to indicate the efficacy of mitigation procedures, such as in-situ burning and dispersant use, thus helping to inform decision makers and net environmental benefit analysis (NEBA) in real time (IPECA, 2016; ITOPF, 2012). Hence, the development of a suite of bio-indicator species and sublethal biomarkers for accurate oil spill assessment has been recommended (Brussaard et al., 2016).

The environmental risk assessment of oil spills should ideally consider environmentally relevant concentrations, encompass hazard data on a variety of species across trophic levels, and include intracellular indicators of sublethal toxicity (Coelho et al., 2013; Galloway et al., 2002). A wide range of biological assays including lethal and sublethal effect endpoints have been recommended for monitoring programs

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https://doi.org/10.1016/j.marpolbul.2021.112151
Received 22 October 2020; Received in revised form 2 February 2021; Accepted 3 February 2021
Available online 15 February 2021

(Blaise et al., 2004). Commercially available toxicity kits that rely on marine organism cysts provide a simple and rapid test system that could be readily implemented in oil spill field monitoring programs. However, the utility of these assays for use in oil spill contexts is unclear given that the relative sensitivity and performance of these bioassays in evaluating toxicity of hydrocarbons and oils is not documented (Concawe, 2018; Wieczerzak et al., 2016). In particular, challenges have been found with maintenance of volatile and semi-volatile chemical concentrations as most test protocols require the use of plastic open well plate systems, resulting in unacceptable chemical loss (>20%) (Smith et al., 2010b).

A toxicity assessment framework is needed to understand the relative sensitivity of the bioassay species, compared to other aquatic species, and to understand the relationship between observed toxicity and hydrocarbon structure. In the present work, toxicity testing of three aromatic hydrocarbons was performed using two common rapid toxicity kits that rely on cyst-based marine bio-indicator species, brine shrimp (*Artemia franciscana*) and marine rotifer (*Brachionus plicatilis*). These bioassays are typically performed in miniaturised systems, e.g. well plates (Snell and Persoone, 1989; Wells et al., 1998), which can result in loss of test chemical and inaccurate test results. Therefore, we employed state of the art passive dosing in sealed test systems to provide reliable data that avoids artefacts due to experimental losses (Bragin et al., 2016).

Passive dosing is a method applicable to hydrophobic organic carbons (HOCs) which allows the accurate dosing of aqueous solutions by forming a partitioning equilibrium of test chemicals from a pre-dosed biocompatible polymer to the aqueous test media (Smith et al., 2010a, 2013). The method does not require any carrier solvents to form a homogenous solution, thus preventing potential co-solvent toxicity (Hutchinson et al., 2006). In addition, potential loss of volatile compounds is compensated by continued partitioning throughout exposures (Smith et al., 2010a, 2013). The method effectively controls and maintains individual hydrocarbon concentrations across a variety of species from varying habitats to investigate oil spill toxicity (e.g. zebrafish (Butler et al., 2016), shallow water corals (Renegar et al., 2017) and deep sea crustaceans (Knap et al., 2017)).

Individual hydrocarbon testing can be used to overcome the complexity associated with assessing oil toxicity, especially when calibrated using the target lipid model (TLM) (Butler et al., 2013). The TLM shows the relationship between a chemical's toxicity and structure (Di Toro et al., 2000; McGrath et al., 2018). The model assumes a critical body burden hypothesis which suggests internal target lipid concentrations dictate toxicity (van Wezel et al., 1995), where target lipid refers to the specific site of action (e.g. lipid membranes). This framework can support comparison of toxicity results from different chemicals that have a similar mode of action, but very different partitioning properties. It has been used to evaluate nonpolar, polar, and ionic molecules (Escher et al., 2019; Kipka and Di Toro, 2009; McGrath et al., 2018; Redman et al., 2017) and represents an interesting framework for comparing the relative sensitivity of different aquatic species. Furthermore, the TLM and similar frameworks have been extended to modelling the toxicity of complex crude oils, and other refined oil substances enabling these data to be compared to whole oil tests (French-McCay, 2002; Macleod et al., 2004; McGrath et al., 2005; Olsen et al., 2013; Redman et al., 2012, 2014).

The specific objectives of this study were to develop reliable exposure and toxicity data for two miniaturised marine toxicity tests and evaluate the relative sensitivity of these bioassays using the TLM. This was achieved through the application of a miniaturised, closed-vial, passive dosing system to assess the toxicity of three representative aromatic hydrocarbons (AH) with varying physicochemical characteristics; toluene, 1-methylnaphthalene and phenanthrene. Miniaturised, cyst-based marine bio-indicator species tests (brine shrimp (*Artemia franciscana*), and marine rotifer (*Brachionus plicatilis*)) were used. Our multidisciplinary approach and careful validation allowed the discussion of the application of these bioassays applicability to in-situ oil spill biomonitoring.

2. Materials and methods

2.1. Chemicals and materials

Chemicals were obtained from various suppliers: ethyl acetate (99 + %, Acros Organics), methanol (MeOH) (99.8%, Fisher Chemical), pentane (95%, Rathburn), toluene (99.8%, Alfa Aesar), 1-methylnaphthalene (96%, Acros Organics), and phenanthrene (98%, Alfa Aesar).

Glassware was acid washed by submersion in a 10% nitric acid bath for 12 h and rinsed 3 times with deionised water (dH_2O) before first use and rinsed 3 times with methanol (MeOH) before every use. All glassware used for GC–MS analysis was rinsed 3 times with pentane and left to evaporate prior to use.

2.2. Preparation of PDMS for passive dosing

Silicone, polydimethylsiloxane (PDMS), O-rings (6 mm diameter, 1.5 mm thickness, 0.034 g) were obtained from Barnwell (Bristol, UK). The O-rings were cleaned of any impurities before use by rinsing in a 2 L Erlenmeyer flask with ethyl acetate (overnight), MeOH (3 times in 24 h) and dH_2O (3 times in 24 h). Prior to use O-rings were dried in an oven at 80 °C. Clean and dried O-rings were stored in a glass beaker covered with foil until required.

2.3. Loading of PDMS

Aqueous concentrations were dosed and maintained throughout exposures by adapting the PDMS passive dosing method described by Butler et al. (2013). Briefly, PDMS O-rings were loaded with a stock solution of AH (toluene, 1-methylnaphthalene or phenanthrene) in MeOH using the equation:

$$C_{MeOH} = \left(\left(K_{MeOH:PDMS + \left(\frac{V_{PDMS}}{V_{MeOH}}\right)} \right) \times \left(K_{PDMS:Seawater + \left(\frac{V_{Seawater}}{V_{PDMS}}\right)} \right) \right) \times C_{Nominal}$$

where C_{MeOH} is the concentration of added to MeOH (mgL^{-1}); $C_{Nominal}$ is the desired concentration in seawater (mgL^{-1}); V_{MeOH} is the volume of MeOH (mL); V_{PDMS} is the volume of PDMS O-rings in the MeOH dosing vessel or aqueous media (mL); V_{water} is the volume of water in the toxicity test exposure system (mL); $K_{MeOH:PDMS}$ is the partition coefficient of the AH between MeOH and PDMS; and $K_{PDMS:Seawater}$ is the partition coefficient of the AH between PDMS and artificial sea water (35 ppt (± 1) at 25 °C (±1)) (Butler et al., 2013).

2.4. Calculating partition coefficients

The specific $\log K_{MeOH:Water}$ for each AH with 6 mm O-rings was determined by dissolving a predetermined amount of each AH in 250 mL MeOH with the required number of O-rings. The O-ring and AH solution was incubated in a shaker for 72 h at 25 °C and 150 rpm. O-rings were removed from the methanol, rinsed three times with deionised water and blotted with lint free tissue being careful to remove all droplets. For calculation of the specific partition coefficient within the 4 mL glass vials, three O-rings were added to each 4 mL vial filled to form a convex meniscus with artificial seawater (35 ppt (± 1) at 25 °C (±1)) and sealed with a Teflon lined cap. Vials were incubated for 24–48 h at 25 °C and in darkness. After 24 h, 3 replicates of each concentration were prepared for GC–MS analysis in 20 mL crimp top headspace vials and stored at

4 °C (±1) overnight. After 48 h, 3 replicates of each concentration were prepared for GC–MS analysis to assess any loss within the system. The method is summarised in Fig. 1.

The biological exposure vessels were passively dosed as above using concentrations based on the species sensitivity shown in preliminary range finding tests. The concentrations were based on known dose response curves for these species to ensure the accurate estimation of LC values enabling comparison to modelled species sensitivity. The number of biological replicates used and total number of O-rings required for all treatments are shown in Table 1.

2.5. Marine toxicity tests

Artemia franciscana, sourced in cyst form from ZM systems (Winchester, UK), were hatched for 30 (±1) hours in 10 mL 35 ppt artificial seawater (Reefmix Sea Salt, Tropic Marin, Germany at 25 °C with 3000–4000 lx. Post hatching, 10 organisms were added to three 4 mL vials of each concentration which had been preloaded with (35(±1) ppt, pH 8.2(±0.2)) three O-rings using a transfer pipette. The vial was closed with no headspace. Organisms were incubated in darkness for 24 h at 25 °C. The survival rate was calculated by pouring the test water from the vials into a 12 well plate and observing each replicate under a microscope (×10 magnification), organisms were counted and were considered dead if no movement was observed after 10 s. Tests were accepted where control mortality was below 10%.

Brachionus plicatilis, sourced in cyst form from Microbio Tests (Gent, Belgium), were hatched for 37 (±1) hours in 5.7 mL 35 ppt artificial seawater and 4.3 mL distilled water at 25 °C with 3000–4000 lx. Post hatching, 5 organisms were added to five 4 mL vials of each concentration which had been preloaded with artificial seawater (35 ± 1 ppt, pH 8.2 ± 0.2) and three O-rings using a transfer pipette. The vial was closed with no headspace. Organisms were incubated and counted as described for the *A. franciscana* tests. Tests were accepted where control mortality was below 10%.

2.6. Chemical analysis

After organisms were introduced (0 h) and counted (24 h) the concentrations of toluene, 1-methylnaphthalene and phenanthrene were measured using GC–MS via headspace sampler. Chemical control samples were prepared in triplicate as close to the start and end of the biological exposures as possible (±3 h). Samples were diluted in deionised water and spiked with 40 μgL^{-1} bromochloro-methane, 1,2-Dichloroethane-d4, 1,4-diflurobenzene, D8-toluene, D5-chlorobenzene, 1-bromo-4-flurobenzene 10 mgL^{-1} internal standard mix in a 20 mL glass chromatography vials. Prepared samples were stored in the refrigerator at 4 °C until analysis. To ensure quality control a minimum of a 5-point calibration was used with an $R^2 > 0.98$. Quality control samples of a known concentration (± 20%) were run every 9 samples to monitor any variance from the calibration. All standards were purchased from SUPELCO, Sigma-Aldrich.

Samples were analysed in selective ion monitoring mode using a GC–MS (Aligent Technologies 7890B GC System and 5977A mass selective detector) fitted with a Restek Rxi-624Sil MS (30 m × 250 μm × 1.4 μm) column and equipped with headspace sampler (Agilent Technologies 7697A). Helium was used as the carrier gas with a flow rate of 1.5 $mLmin^{-1}$. The oven temperature was programmed to an initial temperature of 43 °C and held for 4 min before increasing to 55 °C at a rate of 5 °$Cmin^{-1}$ before immediately increasing to 210 °C at a rate of 15 °$Cmin^{-1}$ and held at this temperature for 5 min. For phenanthrene samples, the method was extended to hold at 210 °C for 25 min.

2.7. Statistical analysis

The mean measured concentration of 0 and 24 h chemical samples and mortality in each vial after 24 h was used to calculate the LC10 and LC50, the concentration of a substance causing death in 10% and 50% of test organisms in both tests. LC values were calculated using the LL.4. Dose Response Model (DRM) function (Ritz et al., 2015) and plotted using GGPLOT in R (R Core Team, 2020). Chemical analysis was tested for normality using the Shapiro-Wilk Test prior to one-way ANOVA. Statistical analysis and plots were performed in GraphPad Prism 8.3.1 (GraphPad Software, LLC) and Microsoft Excel 2013.

Following LC estimation, critical target lipid body burdens (CTLBBs) were calculated by linear regression of Eq. 1, fitting only the logCTLBB, applied to all three test chemicals. This results in the mean logCTLBB across the three test chemicals. This was performed for each of the test species:

$$logLC50 = mlog(K_{OW}) + log(CTLBB) + \Delta c \quad (1)$$

where m is the universal slope (−0.936) as described by Di Toro et al. (2000), K_{ow} is the octanol-water partition coefficient of the chemical, *CTLBB* is the critical target lipid body burden for the species ($\mu molg^{-1}$) derived from the intercept and Δc is the correction factor attributed to the chemical class (0.025 for toluene and 0.364 for 1-methylnaphthalene and phenanthrene) (Di Toro et al., 2000; McGrath et al., 2018).

3. Results and discussion

3.1. Maintenance of chemical concentrations

Achieving accurate maintenance of concentrations using passive dosing relies on the measurement of each chemical's partition coefficient between the PDMS and the water ($K_{PDMS:water}$). For both species used here, tests were conducted at 25 °C (±1) and 35 ppt (±1) and within a closed, small-scale volume of 5 mL. To achieve accurate dosing, partition coefficients were adjusted based on the results of preliminary tests using a known mass of AH to give the expected $LogK_{MeOH:seawater}$ of

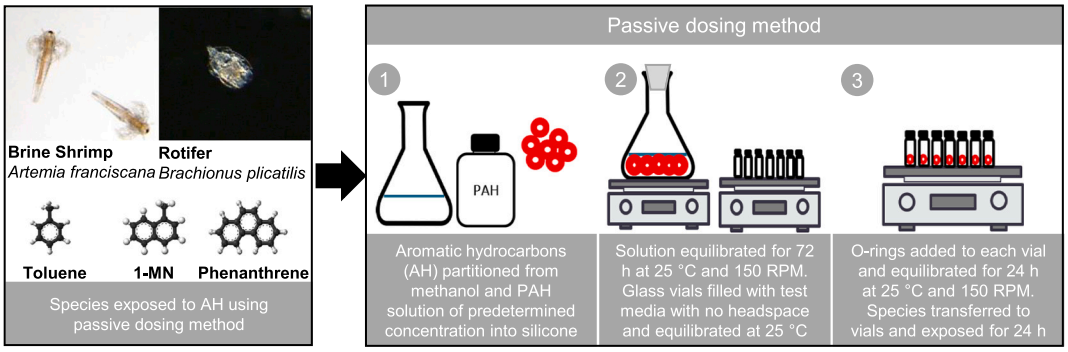

Fig. 1. Summary of dosing and exposure used during this study.
(Adapted from Colvin et al., 2020.)

Table 1
Test replicates and partition coefficients used for toxicity tests.

Species	No. treatment replicates	O-rings added to MeOH	Nominal concentrations (mgL^{-1})		
			Toluene	1-Methylnaphthalene	Phenanthrene
A. franciscana	3	27	0, 15, 30, 45, 60, 100	0, 0.75, 1.5, 2.25, 3, 3.75	0, 0.2, 0.4, 0.6, 0.8, 1
B. plicatilis	5	33	0, 15, 30, 45, 60, 100	0, 1, 2, 3, 4, 5	0, 0.2, 0.4, 0.6, 0.8, 1

2.97 for toluene, 3.58 for 1-methylnaphthalene and 4.27 for phenanthrene.

To monitor exposure concentrations and loss during the exposure, aqueous samples were measured at the start and end of the tests (0 and 24 h). The mean measured $LogK_{MeOH:seawater}$ observed for each of the 5 concentrations were calculated as 2.99 for toluene, 3.56 and 3.66 for *A. franciscana* and *B. plicatilis* 1-methylnaphthalene exposures, and 4.18 for phenanthrene. Measured partition coefficients were similar to the predicted partition coefficients for all three of the test hydrocarbons ($P > 0.8$).

Mean measured concentrations of toluene, 1-methylnaphthalene and phenanthrene compared with expected concentrations at each time point are shown in Fig. 2. The concentrations reached were generally consistent across samples and for the duration of the exposures; measured concentrations after 24 h were within 16% of the initial concentration. Such stability is important for reducing uncertainty related to variability of exposure concentrations that can occur during conventional (e.g., co-solvent) dosing. Previous work has shown passive dosing as a reliable method of maintaining volatile and semi-volatile compounds during exposures in larger, PDMS cast 10 mL and 20 mL vials (compared with the 5 mL volume used here) (Rojo-Nieto et al., 2012; Smith et al., 2010a, 2013), and in larger and flow through test systems (Butler et al., 2016; Knap et al., 2017; Renegar et al., 2017) which can remain stable with respect to exposure concentration for up to 128 h (Smith et al., 2010a).

When combined with chemical analysis to confirm concentrations, passive dosing can thus provide a stable, replicable and solvent free method of dosing, rendering the data suitable for analysis using the TLM framework (McGrath et al., 2018).

Standardised protocols for the rapid assessment of the test species are well established (Snell and Persoone, 1989; Wells et al., 1998), but the open plate format is vulnerable to losses of AH during exposure. The closed passive dosing system used here was designed to prevent such loss. Recent studies have passively dosed PAHs into open 24-well plates for algal exposures (Niehus et al., 2018). An estimated 35% loss was recorded over the 72 h exposure for 1-methylnaphthalene, suggesting the method poses some challenges for assessing more volatile compounds. The use of well plates, or an alternative small, sealed vessel in which the organisms are visible may prevent technical challenges associated with the additional handling required when using closed vials, in particular with rotifers.

3.2. Sensitivity of lethality assays

All control treatments (including un-dosed silicone) for both brine shrimp (*A. franciscana*) and marine rotifer (*B. plicatilis*) showed <10% mortality which is within the acceptance criteria for the test.

The LC50s for both species were similar for each of the three

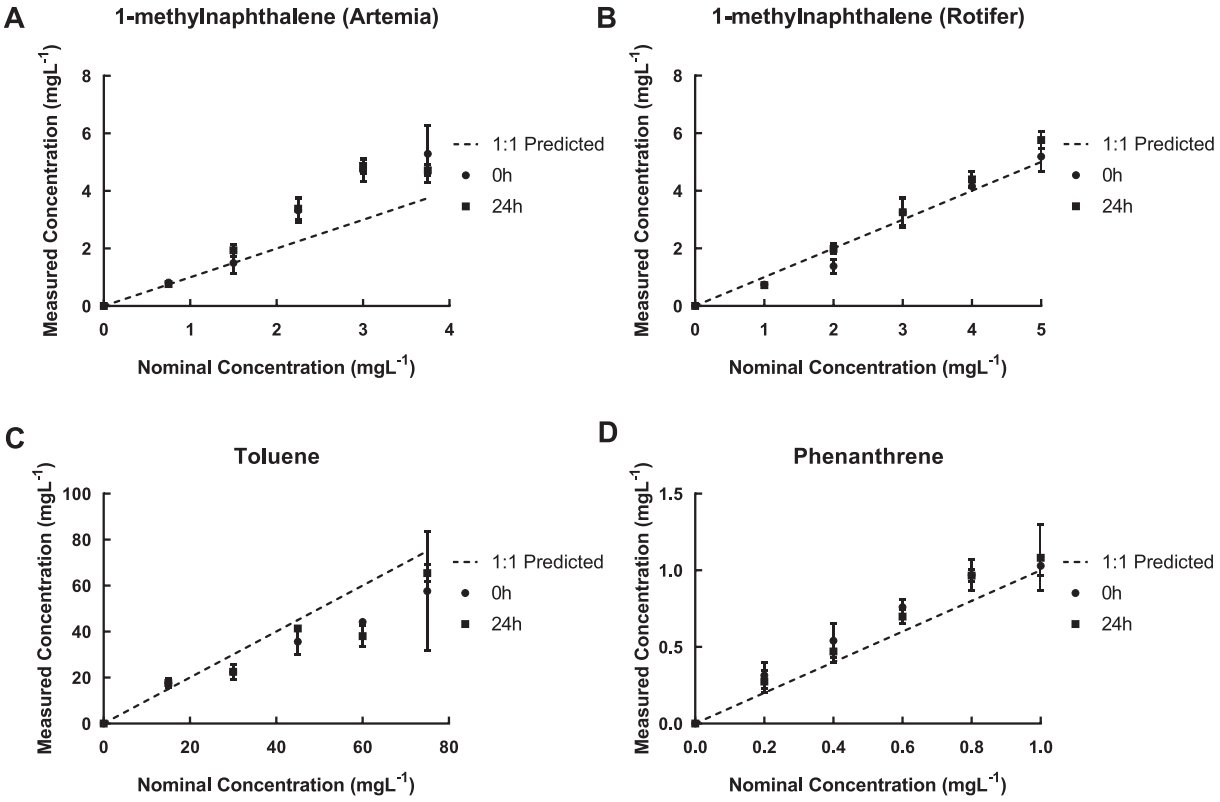

Fig. 2. Measured compared to nominal exposure concentrations of 1-methylnaphthalene (A and B), toluene (C) and phenanthrene (D) at 0 and 24 h (±2) after dosing brine Shrimp (*Artemia franciscana*) (A), marine rotifer (*Brachionus plicatilis*) (B) or, both (C and D) ± SD. The 1:1 predicted line shows the agreement between the nominal and observed concentrations ($n = 3$). Empirical data is shown in Table S1.

hydrocarbons. Higher toxicity was observed for test hydrocarbons in both species with increasing molecular weight consistent with theory (Eq. (1)). Concentration-response relationships are shown in Fig. 3. The LC50 values were used to increase the comparability of these findings to the literature; LC10 values have also been calculated for reference and to highlight the steepness of the observed concentration-response relationship.

The calculated LC50 values for brine shrimp were 22.10 (±0.87) mgL^{-1}, 1.81 (±0.17) mgL^{-1} and 1.00 (±0.02) mgL^{-1} and LC10 values were 14.92 (±1.39) mgL^{-1}, 1.27 (±0.45) mgL^{-1}, 0.80 (±0.05) mgL^{-1} for toluene, 1-methylnaphthalene and phenanthrene respectively. For marine rotifers the LC50 values were 40.65 (±0.54) mgL^{-1}, 3.00 (±1.97) mgL^{-1} and 1.89 (±1.55) mgL^{-1} and LC10 values were 39.79 (±1.50) mgL^{-1}, 2.72 (±3.97) mgL^{-1}, 0.63 (±0.48) mgL^{-1} for toluene, 1-methylnaphthalene and phenanthrene respectively. It was not always possible to count all of the marine rotifer (*B. plicatilis*) within the counting chambers following the 24 h exposure, most likely as a result of loss during the transfer steps from the vial into the counting chamber or vice versa. More total individuals were successfully counted during the reference test using the same method (potassium dichromate = 89% of n = 150, compared to toluene = 53%, 1-methylnaphthalene = 76%, phenanthrene = 65%). In addition, counting chambers appeared to contain more organic material in the AH test media, compared to reference tests with potassium dichromate (Fig. S1). During reference tests, dead rotifers remained visible, suggesting some organisms may have disintegrated in the AH tests. However, despite more individuals being counted in the control media for toluene and phenanthrene, there was no clear dose-response trend between AH concentration and organism loss.

Such technical issues may explain the poor fitting of the dose response model to the phenanthrene data for marine rotifers, as no clear dose response up to 1 mgL^{-1} was observed (Fig. 3F). It was not possible to test higher concentrations due to precipitate formation, suggesting the LC50 value for marine rotifers is above the solubility limit of phenanthrene (1.10 mgL^{-1} at 25 °C in water (Yalkowsky et al., 2016)). These data show that toxicity increased with increasing molecular weight and therefore decreasing solubility (Fig. 4) conforming to similar fits observed elsewhere (Di Toro et al., 2000; McGrath et al., 2018).

3.3. Comparative toxicity

Laboratory to field extrapolations require a model framework that accounts for changes in the magnitude and composition of the exposure, as well as changes in organism sensitivity. By fitting the empirical toxicity data generated in this study to the TLM it is possible to calculate the critical target lipid body burden (CTLBB) from the intercept of the linear regression using Eq. 1. The more sensitive a species is to hydrocarbon exposure, the lower the y-intercept will be (Di Toro et al., 2000; McGrath et al., 2004, 2005, 2018). The linear regressions derived in this study are shown in Fig. 4.

Log(CTLBB)s were calculated using both LC50 for both species resulting in a Log(CTLBB) of 1.97 ± 0.28 for brine shrimp (*A. franciscana*) and 2.23 ± 0.30 for marine rotifer (*B. plicatilis*) which when translated to untransformed values yield CTLBBs of 93.9 ± 11.7 and 168.3 ± 23.4 $\mu mol/g_{octanol}$ based on LC50 (Fig. 4). Thus, brine shrimp is less than a factor of two more sensitive to AH than the marine rotifer. When these CTLBBs are compared to the 79 aquatic species modelled (McGrath et al., 2018) these species-specific endpoints fall in the upper third and fourth quartile ranges (50–75% and 75–100% of most resistant species respectively) of the acute species sensitivity distribution of more resistant species, respectively (Fig. 5).

These results confirm that the organism and effect endpoints have comparable sensitivity to other aquatic organisms with moderate to lower sensitivity. The variability in the CTLBB estimates are similar or smaller than data for other organisms, which reflects the very stable exposure concentrations achieved with passive dosing. The results from the present study show that these simple cost-effective marine bioassays can provide reliable hazard data that contribute to species-sensitivity distributions used in derivation of water quality objectives. However, if used in field assessments such assays would likely understate potential effects on sensitive organisms. Ideally, a toxicity kit used in field monitoring contexts should exhibit a CTLBB corresponding to the 5th percentile of the species sensitivity distribution for assessing potential ecosystem impacts (Posthuma et al., 2001; Versteeg et al., 1999).

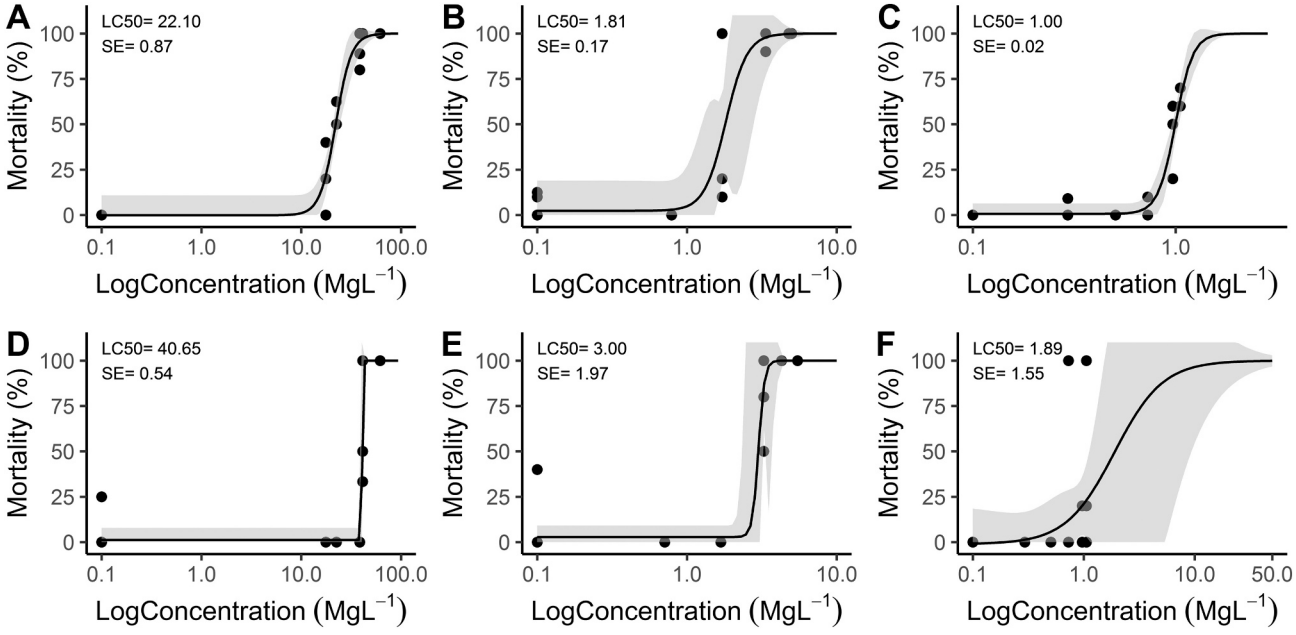

Fig. 3. Effect of A) toluene, B) 1-methylnaphthalene and C) phenanthrene on brine Shrimp (*Artemia franciscana*) and D) toluene E) 1-methylnaphthalene and F) phenanthrene on marine rotifer (*Brachionus plicatilis*) survival rate after 24 h exposure. Black points denote mortality per vial (brine shrimp n = 10/3, marine rotifer n = 5/5), black line shows modelled dose response and grey shaded area represents ±95% CI of the predicted dose response curve. In order to fit the scale, control (0) values have been transformed to 0.1 mgL^{-1} and confidence intervals >0 or < 110 have been capped at 0 and 110 respectively.

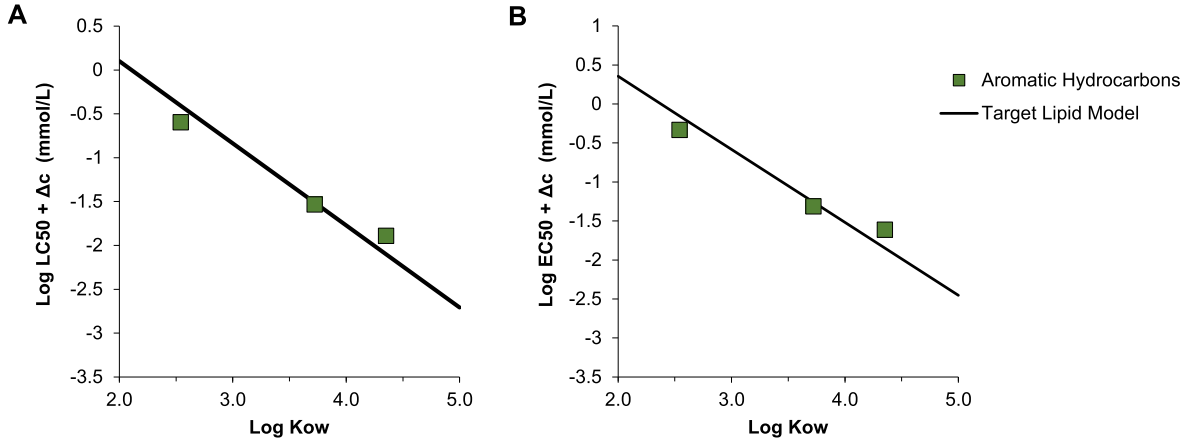

Fig. 4. A) Brine Shrimp (*Artemia franciscana*) and B) Marine Rotifer (*Branchionus plicatilis*) LC50s for aromatic hydrocarbons as a function of substance Log K_{OW}. Measured LC50 data was adjusted using reported class-specific correction factors to allow comparison to the target lipid model, shown by the solid line (McGrath et al., 2018).

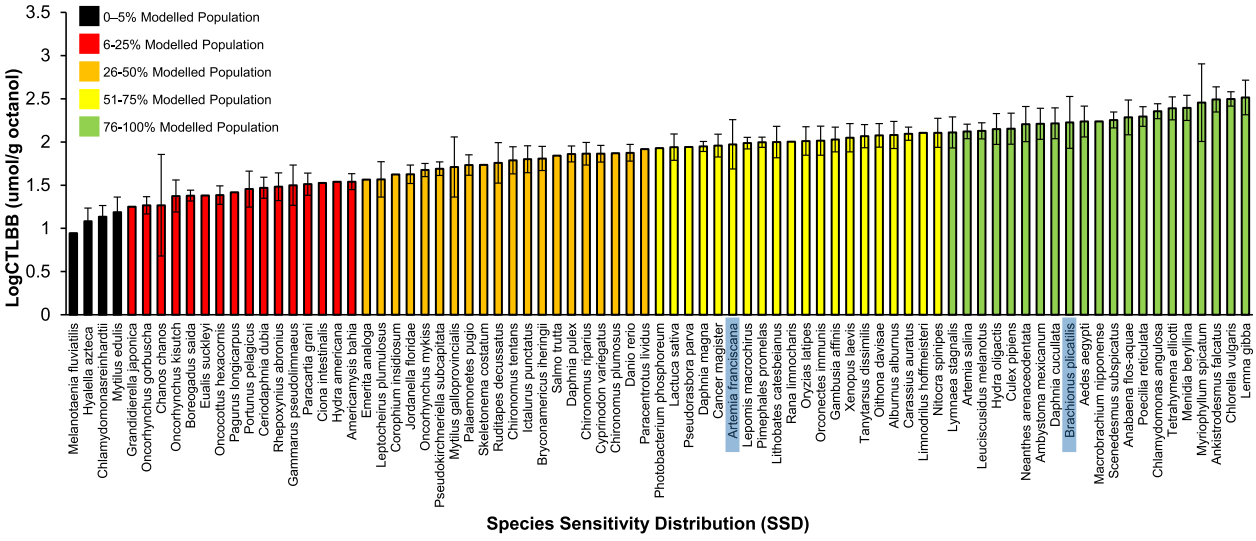

Fig. 5. Relative species sensitivity distribution of marine rotifer *Brachionus plicatilis* and brine shrimp *Artemia franciscana* (highlighted blue) against species modelled using the target lipid model (TLM) to date (McGrath et al., 2018). Bars represent the relative critical target lipid body burden Log(CTLBB) (± SE) of each species calculated using the TLM. Colours represent the percentile ranges of the modelled community from most to least sensitive: black = 0–5%, red = 6–25%, amber = 26–50%, yellow = 51–75% and green = 76–100%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Future work could include testing of whole oils to validate toxicity predictions using the TLM-based PETROTOX model (Redman et al., 2012, 2017). PETROTOX combines the TLM and hydrocarbon block method to predict oil toxicity based on the chemical composition of the test oil. This spreadsheet model groups hydrocarbons of a similar carbon number and structure (and therefore solubility) based on the assumption they will have similar environmental distribution and fate (King et al., 1996). Species specific toxicity can be estimated by dividing the predicted dissolved oil concentration of the hydrocarbon block by the species-specific LC50 that is derived from the calibrated TLM using single hydrocarbon toxicity data, as illustrated in this study, to compute a toxic unit (TU). Assuming each block contributes additively to toxicity, a 50% response is expected when the sum of TUs equals one. Such predictions can be evaluated by comparing PETROTOX predictions to empirical oil toxicity data (Redman et al., 2017). Thus, future empirical toxicity testing with different oils or petroleum substances of known composition using the two miniaturised marine assays would be a logical extension of the present research to further validate such predictive models.

3.4. Applicability to oil spill and environmental assessment

To assess the potential applicability of these rapid, miniaturised biological tests to environmental assessment following an oil spill the environmental relevance of the species sensitivities must be considered. For example, Deepwater Horizon, the most recent and largest oil spill to date, resulted in 507 million litres of Macondo crude oil being spilt in the Gulf of Mexico (U.S. District Court for the Eastern District of Louisiana, 2010). Of 11,407 offshore sites monitored following Deepwater Horizon 39% found no detectable total PAH concentrations while less than 30% of samples collected exceeded reported background concentrations of 0.03 to 0.4 μgL^{-1} and higher in seep areas. Some (0.2% of samples) measured during the early phases of the spill events and prior to any oil recovery from the wellhead measured more than 100 μgL^{-1}. With the exception of surface slicks, high concentrations were less than 2 km from the wellhead, the highest concentration of 151,000 μgL^{-1} was measured

at the well head during the release (Boehm et al., 2016). Chemical dispersants were used directly at the well head at a depth of 1522 m following the spill, to promote dissolution and small droplet formation (Hemmer et al., 2011). This application technique reduced the quantity of oil that made it to the surface. However, a hydrocarbon rich plume at ~1000 to 1200 m depth containing more than 50 μgL^{-1} benzene, toluene, ethylbenzene and total xylene (BTEX) across an area of up to 16 km from the well was reported (Camilli et al., 2010).

Such hydrocarbon concentrations in marine environments are not limited to large scale spills. The *North Cape* barge spilt ~3 million litres of fuel oil off the coast of Rhode Island in 1996 (Ho et al., 1999). Gale force winds (100 km/h) and sea state (5 m waves) resulted in natural oil dispersion throughout the water column ranging to 115 and 3940 μgL^{-1}, some of the highest documented following a spill (Reddy and Quinn, 2001). However, the observed concentrations in grab samples collected following a spill are expected to be highly variable and often fall rapidly. Following Exxon Valdez, a tanker spill which resulted in 41 million litres of crude oil being discharged into Prince William Sound, Alaska, mean total PAH concentrations ranged from 0.27 ± 0.32 μgL^{-1} within 1 m of the nearshore surface, 0.19 ± 0.27 μgL^{-1} within 1 m of the surface further offshore and 0.11 ± 0.11 μgL^{-1} at 3–5 m two months after the spill (Neff and Burns, 1996). The low observed concentrations can be explained by the rapid dilution that occurs in the ocean environment (Lee et al., 2013). Given that AH concentrations reported in field spills are typically in the ppb range, the miniaturised tests investigated in this study are unlikely to serve as an effective field monitoring tool. Thus, until more sensitive bioassays are identified and validated, field monitoring efforts should focus on improved analytical characterization of dissolved oil exposures (National Academies of Sciences, 2020).

Hydrocarbon contamination is not only a risk in an oil spill scenario. BTEX and PAHs are also included in the EU list of priority substances and must be monitored according to the European Union Directive 2013/39/EU (European Union, 2013). A recent review of studies monitoring priority substances and contaminants of emerging concern by Sousa et al. (2018) found that five studies measured the concentrations of naphthalene in surface waters finding mean concentrations of 0.001 mgL^{-1} and ranging from 5 ngL^{-1} to 0.005 mgL^{-1} while 13 studies measured mean total PAH of 0.005 mgL^{-1} which ranged from 5 ngL^{-1} to 0.06 mgL^{-1}. Such in-situ exposure concentrations should be borne in mind when considering the utility of the two acute marine tests considered in this work as a biomonitoring tool for routine water quality monitoring.

The bioassays investigated in this study are particularly suitable to field deployment due to their small size, portability, ability to store cysts and perform tests on short notice. However, the typical format of these tests involve open test systems which compared to the constant exposures achievable in the laboratory may create some uncertainty in interpretation of field testing. The present study has reduced this uncertainty by characterising the relative sensitivity of the tests under well-defined conditions. Comparison of toxicity results to other species indicate these bioassays are unlikely to detect the low-level exposures that have been reported in the field following oil spills or in routine water quality monitoring programs. Thus, more sensitive bioassays that correspond to the 5th percentile sensitivity and/or chemical analysis methods that target dissolved oil (Redman et al., 2018) are needed to support risk assessment of exposures in the field.

4. Conclusions

This work demonstrates passive dosing using O-rings to be an effective method to reach and maintain AH exposure concentrations volumes as small as 5 mL. These findings support and extend previous work such as Butler et al. (2016, 2013) and Smith et al. (2013, 2010a, 2010b) and suggest O-rings provide a reliable and simple method of passive dosing across a variety of exposure vessel volumes, negating the requirement for a silicone cast. The application of the TLM demonstrated that these bioassays result in predictable results (e.g. LC50s scale with logKow). The TLM-derived CTLBBs demonstrate that these bioassays have similar levels of sensitivity with variability that is similar to other test organisms.

The TLM has been broadly applied to a wide range of species across trophic levels and habitats and provides a basis for comparison between species, but also a basis for performing risk assessments on complex substances, like oils. Thus, application of these bioassays within the context of the TLM-derived thresholds indicate that the two tests investigated would have limited utility in supporting decision-making during spills or for routine water quality monitoring.

CRediT authorship contribution statement

Katherine Colvin: Methodology, Investigation, Formal analysis, Writing- Original Draft, Visualisation. **Tom Parkerton:** Conceptualisation, Writing- Review & Editing. **Aaron Redman:** Conceptualisation, Writing- Review & Editing. **Ceri Lewis:** Writing- Review & Editing, Supervision. **Tamara Galloway:** Conceptualisation, Writing- Review & Editing, Supervision.

Declaration of competing interest

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

Acknowledgements

With thanks for the expertise and guidance from the Concawe Special Task Force on Biological Effects/ Measures (STF-32), and Rob Holland from OSRL. Thanks also to Greenpeace Research Laboratories for their technical advice and use of their GC–MS instrumentation.

Funding

This work was supported by the University of Exeter, United Kingdom, European Petroleum Refiners Association (Concawe), Belgium, and Oil Spill Response Ltd., United Kingdom. Ref: 201700038.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2021.112151.

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