Published in final edited form as:
Aquat Toxicol. 2023 June ; 259: 106518. doi:10.1016/j.aquatox.2023.106518.

Recommendations for advancing media preparation methods used to assess aquatic hazards of oils and spill response agents

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Abstract

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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.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2023.106518.

Laboratory preparation of aqueous test media is a critical step in developing toxicity information needed for oil spill response decision-making. Multiple methods have been used to prepare physically and chemically dispersed oils which influence test outcome, interpretation, and utility for hazard assessment and modeling. This paper aims to review media preparation strategies, highlight advantages and limitations, provide recommendations for improvement, and promote the standardization of methods to better inform assessment and modeling. A benefit of media preparation methods for oil that rely on low to moderate mixing energy coupled with a variable dilution design is that the dissolved oil composition of the water accommodation fraction (WAF) stock is consistent across diluted treatments. Further, analyses that support exposure confirmation maybe reduced and reflect dissolved oil exposures that are bioavailable and amenable to toxicity modeling. Variable loading tests provide a range of dissolved oil compositions that require analytical verification at each oil loading. Regardless of test design, a preliminary study is recommended to optimize WAF mixing and settling times to achieve equilibrium between oil and test media. Variable dilution tests involving chemical dispersants (CEWAF) or high energy mixing (HEWAF) can increase dissolved oil exposures in treatment dilutions due to droplet dissolution when compared to WAFs. In contrast, HEWAF/CEWAFs generated using variable oil loadings are expected to provide dissolved oil exposures more comparable to WAFs. Preparation methods that provide droplet oil exposures should be environmentally relevant and informed by oil droplet concentrations, compositions, sizes, and exposure durations characteristic of field spill scenarios. Oil droplet generators and passive dosing techniques offer advantages for delivering controlled constant or dynamic dissolved exposures and larger volumes of test media for toxicity testing. Adoption of proposed guidance for improving media preparation methods will provide greater comparability and utility of toxicity testing in oil spill response and assessment.

Keywords

Oil toxicity testing; Media preparation; Water accommodated fraction; Passive dosing

1. Introduction

Aquatic hazard assessment is a critical aspect of spill response decision-making (Bejarano et al. 2014; Bi et al. 2021; USEPA 2021; Walton et al. 2021). Relevant test substances include crude oils, petroleum products, spill response agents as well as constituents that comprise these substances. Based on oil density, crude oils are classified into light, medium, heavy, and extra heavy, e.g diluted bitumens, categories. Light oils have low viscosities and are comprised of hydrocarbons that are more water soluble. As crude oil density and viscosity increases, the hydrocarbon components become less soluble. Petroleum products are produced by refining crude oils and consist of more narrow distillation cuts of mostly saturated and/or unsaturated hydrocarbons. Examples include gasoline, kerosene, diesel, heavy fuel oil, lubricants, and hydrocarbon solvents (CONCAWE, 2020). Spill response agents, also referred to in some jurisdictions as spill treating agents (Brown et al. 2011), include dispersants, bioremediation, surface washing, and surface collecting (i.e., herders), and miscellaneous agents (USEPA 2021). The preparation method used to introduce these substances into aqueous test media is a key aspect of characterizing the toxicity of these substances to aquatic organisms.

Media preparation with oils is particularly challenging due to the complex composition of crude oil and petroleum substances and the different aqueous solubility, volatility, and biotic and abiotic degradability of individual compounds that comprise these substances. Multiple media preparation methods have been reported and applied for toxicity testing with oils (Adams et al. 2017; Michelmore et al. 2020a). The method chosen has important implications that can influence the bioavailability and consistency of test substance exposures and resulting toxicity (Redman & Parkerton, 2015; Nordtug & Hansen, 2021; Wade et al. 2022). The Chemical Response to Oil Spills: Ecological Effects Research Forum (CROSERF) developed standardized protocols for contaminating test media with physically and chemically dispersed crude oils to promote consistent and comparable aquatic hazard data relevant to acute, marine oil spill scenarios (Aurand & Coelho 2005). However, the lack of consistent application and documentation to an increasing universe of products and spill scenarios coupled with subsequent protocol modifications have challenged interpretation and comparisons across studies (Hodson et al. 2019). Modifications have been implemented for multiple reasons including reducing testing effort, limiting analytical costs, conserving test oil, addressing alternate spill scenarios, and addressing specific research questions.

In addition to traditional hazard assessment (Stubblefield et al. 2023), a recommended future objective of oil spill related toxicity tests is calibrating or validating toxicity models which, when coupled to oil spill fate models, provide predictive tools that support response decision-making (NASEM 2020). Such integrated models are essential since it is impossible to conduct lab toxicity tests that reflect all release scenarios and resulting field exposures following a spill. Dissolved oil exposures are often considered the key focus in toxicity modeling since the freely dissolved form of a substance correlates to chemical activity and is by definition bioavailable (Mackay et al. 2011). Moreover, dissolved oil exposures serve as the technical basis for higher tier models that predict oil spill impacts or compare risks of different response options to aquatic life (French-McCay et al. 2023). Thus, understanding how the media preparation method influences dissolved exposures is crucial in evaluating various methods and linking resulting lab toxicity data topredictive field models.

In contrast, the role of oil droplets on toxicity is less well understood (Hansen et al. 2019a) and is in part due to the lack of practical methods to discriminate particulate oil from dissolved forms (Hodson et al. 2019). Droplet ingestion by zooplankton can influence oil fate and may contribute to adverse effects by reducing feeding rates (Almeda et al. 2014; Hansen et al. 2017) or increasing the potential for phototoxicity (Almeda et al. 2016, Alloy et al. 2022). Oil droplets may adhere to fish gills or embryos and potentially increase localized dissolved oil exposures due to droplet dissolution (Ramachandran et al. 2004; Sørhus et al. 2015; Laurel et al. 2019). A further complication is that reported oil droplet fouling-related effects on fish embryos appear to be species-specific (Carls et al. 2008; Sørensen et al. 2017). Undissolved oil may also cause smothering of respiratory surfaces or physical effects such as entrapment at the water surface (Merlin et al. 2011; Black et al. 2021). To improve understanding and environmental relevance of the potential role of droplets in contributing to oil toxicity, the concentration and stability of oil droplets, size distributions, and composition needs to be considered when applying various media preparation methods. Efforts to quantify droplet exposures and potential effects

of undissolved oil on aquatic life are discussed further by Dettman et al. (2023) and Stubblefield et al. (2023).

The objectives of this paper are to: (1) provide a broad overview of media preparation methods relevant to oil spill contexts; (2) highlight advantages and limitations of CROSERF and alternate methods in providing controlled test substance exposures; (3) provide recommendations for improving future implementation of CROSERF methods, and (4) identify media preparation methods that facilitate toxicity model development and/or validation. Given this later goal, this review expands the scope of the original CROSERF initiative to different spill contexts beyond surficial spills in the pelagic offshore marine environment.

2. Overview of methods for preparing aqueous exposure media

A generic description of media preparation methods is provided in Figure 1 and involves three elements: dosing approach which describes how the substance is added into the test media; dosing regimen which defines if a fixed or variable test media volume is used; and exposure regime which addresses how test organisms interact with the prepared media. Traditionally, test substances have been introduced to aqueous media via direct addition. In the case of hydrocarbon liquids, spill response agents, and oils, the liquid test substance is typically used directly in spiking. For hydrocarbon solids, test substances are typically dissolved in a low toxicity solvent, which serves as a stock solution for test media dosing. In an attempt to develop controlled exposures and prevent co-solvent toxicity (Hutchinson et al. 2006), there has been a growing use of indirect (i.e., passive) dosing methods. These methods add relatively insoluble substances (e.g., hydrocarbons, oils) in or unto a donor medium (e.g. sand or rock substrate, silicone polymer) which is then placed in and used to dose the surrounding aqueous test media. The most common dosing regimen applied for both direct and indirect approaches is static test systems without or with media renewals. If test organisms are transferred to clean media to evaluate potential latent effects after exposures cease, such test designs can be viewed as an on/off pulse exposure that reflect a square wave exposure regime (Michelmore 2020a).

Static test systems are intended to provide constant substance exposures over the test duration. Dosed media can be prepared daily or at appropriate times to allow periodic renewals of test media during toxicity tests if unacceptable declines in exposures are observed. Based on OECD test guidelines for aquatic toxicity testing, a <20% decline in concentration over 24 h for a mono-component substance is considered acceptable (OECD 2019). Achieving this requirement is particularly challenging for oils that contain more volatile and biodegradable components. The inability to maintain consistent exposure concentrations complicates the interpretation of toxicity data between studies and for modeling purposes (Parkerton et al. 2023). A logical strategy to help address this challenge is to focus testing on partially weathered oils in which volatiles (e.g., BTEX and more volatile components) have been removed. Further use of such oils is expected to better reflect actual water column exposures in the field following most surface spill scenarios where volatile components will be rapidly lost (Gros et al. 2014; French-McCay et al. 2023).

To provide consistent initial exposures, dosed test media should be prepared in a manner that ensures all solutions used for renewals exhibit the same age.

Flow-through dosing regimens have also been used with either once-through (i.e., flow-to-waste) or recirculating flows. Once-through flow systems are often designed to provide a continuous exposure, although an alternative approach is a spiked, declining exposure scenario (i.e., initial dosed test solution diluted with clean test media), or dynamic exposures (i.e., initial clean test media dosed intermittently with contaminated solution/clean media) of a test substance. Recirculating systems are generally coupled to indirect dosing methods and are intended to provide continuous exposures. It is important to note that closed (i.e., sealed to prevent loss of volatile compounds) or open test systems may be employed for both static and flow-through dosing regimens. Similarly, subsequent test organism exposures to prepared media may be conducted in open or closed test chambers. The volume of headspace used in closed dosing and exposure systems can also influence dissolved exposures of volatile compounds (Hodson et al. 2019). Examples of studies involving different media preparation methods (Table 1) are discussed further in the next sections.

2.1. Direct addition / static or static renewal methods

Given the water-miscible nature of dispersants and some spill response agents, direct addition with or without renewal is a common media preparation strategy for these products. Separate test treatments may be prepared at different nominal concentrations. Alternatively, a stock solution (e.g., 0.1 to 100 mg/L, based on agent miscibility) can be prepared by physically mixing the test substance in and then diluting in control media to prepare a range of test substance concentrations for toxicity testing, i.e., variable dilution method. A wide variety of direct addition methods have been reported (Table S1) for spill response agents that may result in aqueous exposures containing components in both dissolved and micellular forms if surfactants are components in the product formulation. Depending on the product and spiking concentrations used, test media may represent a water accommodated fraction (WAF) – see section 2.1.1 (Hansen et al. 2014; Echols et al. 2019; Walton et al. 2021). The influence of media preparation method on the observed toxicity of these substances has received little attention, and further comparison and standardization of direct addition methods for these products is needed (Hodson et al. 2019).

Generation of reliable toxicity data for individual hydrocarbons represents an important research priority for advancing oil spill effect models (French-McCay et al. 2023). As a result, media preparation methods for these substances are included within the scope this review. For individual hydrocarbons that exhibit limited water solubility, direct addition can be problematic since resulting exposure concentrations often are not stable and decline rapidly due to volatilization, sorption, and degradation loss processes (Kiparissis et al. 2003). In addition, direct spiking can result in test concentrations that exceed aqueous solubility, thus complicating test interpretation. Flow-through or indirect addition methods are often preferred for preparing media with hydrocarbons to provide more consistent, dissolved exposures (see sections 2.2 and 2.3).

2.1.1. WAF method—The most common preparation method for oils is based on the CROSERF protocol in which test oil is added directly to test media in an aspirator bottle with a specified headspace. The bottle is then sealed and mixed using a stir plate and magnetic stir bar for a set time period. (Singer et al. 2000; Aurand & Coelho, 2005). For less viscous oils, oil can be added using a gas-tight syringe. For more viscous oils, the test substance can be placed in a weigh boat and then transferred to the mixing vessel. The precise amount of oil added can be determined gravimetrically by the mass difference in syringe or weigh boat before and after transfer (Forth et al. 2017a). At the end of the mixing period, stirring is stopped to allow for a prescribed settling period. The resulting water accommodated fraction (WAF) is then sampled and used for aquatic toxicity testing. The approach is intended to and shown to provide stable test media with measured concentrations representing mostly dissolved, bioavailable components of the test oil with limited oil droplets (Sandoval et al. 2017).

To deliver consistent, reproducible dissolved oil exposures from WAFs, test variables defining WAF preparation protocols need to be optimized to achieve specific objectives (Table 2). The original CROSERF protocol was based on the variable loading approach. In this method, each treatment for toxicity assessment is prepared at a different nominal oil loading (i.e., g_{oil} / $L_{test\ media}$). As a result, the dissolved oil composition will reflect the multicomponent dissolution behavior of the oil and thus varies across treatments (Shiu et al. 1988). In contrast, the variable dilution approach relies on preparing a WAF stock at a single oil loading and then making dilutions that serve as treatments for toxicity assessment (Barron & Ka'aihue, 2003).

The time to achieve equilibrium for oil component dissolution into the test media depends on multiple factors, including the oil type and weathering state, mixing energy and duration, oil loading, temperature, test media and test vessel dimensions (Saeed et al. 1998; Tsvenko et al. 2002, Faksness et al. 2008, Zhao et al. 2016; Daskiran et al. 2020; Bilbao et al. 2022). The original CROSERF preparation method for preparing physically dispersed oil involved an 18 h low energy (no vortex) mixing period followed by no settling period and was adopted as a compromise between operational ease and precision in approaching equilibrium conditions (Singer et al. 2000). Further, the short mixing period was selected to minimize potential losses associated with biodegradation and resulting compositional changes with time. Figure 2 illustrates visual differences in WAFs prepared using moderate (20–25% vortex) or high energy (> 25% vortex) mixing with addition of a dispersant at an oil loading of 1 g/L using a light and extra heavy crude oil during mixing (A) and after a 4 h settling period (B).

The predicted influence of mixing energy on droplet size distributions in WAFs prepared using a 4 L aspirator bottle for the two example test oils after 30 min stirring using a magnetic stirrer with a 3.8 cm stirbar was simulated using a numerical model (VDROP) for turbluent regimes based on earlier work by Daskiran et al. (2020). This mixing duration is expected to be sufficient to achieve a steady state oil droplet size distribution. A brief description of these calculations is provided in Appendix S1 along with the oil properties used as model inputs. Mixing energy was expressed as stir bar revolutions per minute (rpm). For reference, stirring speeds at or below ca. 250 rpm are predicted to generate a

negligible vortex while speeds of ca. 700 rpm yield a 20–25% vortex. However, mixing speeds needed to produce a vortex will vary depending on the vessel and stir bar size and should be calibated within each laboratory (Echols et al. 2016a). Modeling shows that as the rpm increases, smaller droplets are generated for both oils, and at the same mixing energy, smaller droplets are predicted for the lighter, less viscous Alaska North Slope crude oil than for the heavier, more viscous diluted Cold Lake Bitumen Blend (Figure 3). Simulations also indicate that the oil size dropet distibution becomes narrower as mixing energy increases as evidenced by the reduced range between 10^{th} and 90^{th} percentile oil droplet sizes.

The oil droplet size distribution obtained at the end of mixing influences the required settling period to remove oil droplets given the typical aim to generate test media reflecting dissolved oil exposures (Table 2). The approximate rise time for different oil droplet sizes can be simulated using Stokes Law, assuming no turbulence once mixing is stopped (Nordtug and Hansen, 2021). Figure 4 shows the modeled rising time for oil droplets exhibiting different densities and surfacing over a 10 cm depth. Results indicate that a linear log-log function describes the relationship between rising time and oil droplet size. Less dense oils exhibit faster rise times due to the action of greater buoyant forces. For the oils simulated, oil droplets with diameters above 10 and 30 microns would be removed from a 10 cm depth in 10 and 1 h, respectively. For a 4 L vessel the depth would be approximately 30 cm so the rise times can be scaled by multiplying by three. Compared with the median droplet sizes predicted in Figure 3, short settling times (e.g., 1 h) are likely sufficient to remove the median size oil droplets generated in typical WAF test systems using magnetic stirring. However, low amounts of small droplets included in the lower tail of the droplet size distribution appear to remain in test media based on both experimental measurements (Ji et al. 2022) and oil solubility modeling (Redman et al. 2012; 2017) despite the clear appearance of settled WAFs (c.f. Figure 2B).

Given that multiple factors influence time to achieve equilibrium between dissolved and droplet oil, a performance-based approach to WAF preparation is recommended by conducting a preliminary study to optimize the procedure prior to toxicity testing using a given oil and WAF test system as illustrated by Bilboa et al. 2022. The objective of this initial work is to provide a method that delivers stable dissolved oil exposures consistent with original CROSERF (Aurand & Coelho, 2005) and subsequent OECD (2019) guidance. An example study design is outlined in Table 3. In contrast to the original CROSEF protocol, moderate mixing energy is suggested to better facilitate equilibrium of dissolved oil components and a sufficient settling period included to more effectively limit droplet exposures. This preliminary study can also be used to determine the stability of dissolved oil exposures after settling to decide the need and frequency of renewals (Bilboa et al. 2022). WAFs should be prepared in closed mixing vessels with a clearly reported headspace, i.e., <20% of the vessel volume (Hodson et al. 2019). Typically, WAF should be drawn from the bottom of the mixing vessel to avoid sampling undissolved oil that typically floats. However, when evaluating heavy oils that sink, WAFs should be sampled from the surface (Girling et al. 1994). If multiple temperatures are used, it is suggested to perform the preliminary WAF study at the lowest temperature, which is expected to have the slowest dissolution kinetics. The intent of this guidance is to provide flexibility in selecting of WAF parameters so that different labs can achieve exposures that reflect equilibrium between the test oil

and media with the equipment and specific laboratory conditions being used. Thus, the aim is to provide more consistent oil exposures that facilitates comparison of toxicity data between labs for the same test oil and support exposure and toxicity modeling of dissolved oil as discussed in Parkerton et al. 2023. It is important to point out than during oil spills in the field, the selected oil loading and equilibrium concentrations of dissolved oil are unlikely achieved. For example, an oil slick with an average thickness of 1 mm has an oil to water ratio of 1:10,000 for the top 1 m of the water column. Further kinetic constraints on oil dissolution and loss processes result in non-equilibrium conditions (McAuliffe et al. 1987). Thus, modeling is required to translate observed effects observed under equilibrium exposures in the lab to dynamic field conditions as detailed by French-McCay et al. 2023.

Based on studies compiled in the review by Adams et al. (2017), the most common oil to water loading reported for physically dispersed oil tests was 1:9 with a range of 1:4 to 1:1000 (ca. <1 to > 250 g/L depending on oil density). Previous work indicates that WAF saturation of PAHs often occurs at loadings of 1–10 g/L for crude oils (Hokstad et al. 1999; Hook & Osborne, 2012; Forth et al. 2017a). However, much higher oil loadings may be required before test media is saturated with the more soluble components, including benzene, toluene, ethylbenzene, and xylenes (Bobra 1992). For aquatic hazard assessment, the goal is not to saturate the test media with specific oil components but rather to select a maximum oil loading that is high enough to produce oil exposures causing obvious toxicity. This approach limits the amount of test oil required, avoids unnecessary waste generation (see section 3.0), and ensures dose-response relationships can be investigated at lower exposure treatments. Based on several studies (Redman et al. 2017; Forth et al. 2017a; Faksness et al. 2020) and modeling (NASEM, 2020) a 1–2 g/L oil loading may be sufficient to generate dissolved oil exposures that are toxic for many crude oils and petroleum substances. However, higher loadings may be required for light, fresh oils and/or less sensitive species/effect endpoints (Barron et al. 2020). The selected loading(s) used in media preparation should be evaluated as part of the screening study discussed above to assess if resulting exposures are sufficiently high to cause adverse effects for the test organism/endpoint being investigated. For oils that are comprised of insoluble components (e.g., artificially highly weathered crude oils, lubricant oils), these substances may not be toxic at extreme loadings so limit studies performed a single high loading (e.g., 10 g/L) may provide a logical strategy to avoid unnecessary testing.

Variable loading tests provide multiple dissolved oil exposure composition profiles with each test treatment that better reflect the variability of spill exposures in the field where the ratio of oil to water varies markedly in space and time (Shiu et al. 1988). However, variable loading tests have the disadvantage that analytical verification is required for each test loading so that the different oil component concentrations and compositions can be quantified. If droplet exposures can be effectively minimized by judicious selection of WAF test protocol variables, the variable dilution protocol offers practical advantages (Hodson et al. 2019). These advantages include that only a single WAF needs to be prepared and less analytical verification may be required. In variable dilution tests, analytical measurements of stock WAF and a subset of test dilutions can be used to confirm the expected linear decrease and stability of exposure concentrations over the test period. If measured concentrations are consistent with predictions based on dilution, these results can be used to estimate the

concentration and composition of dissolved hydrocarbons in remaining test dilutions that are not analyzed. Further, conducting multiple variable dilution toxicity tests with different WAF stocks prepared at alternate loadings can be used to provide toxicity data for different dissolved oil compositions. Thus, testing physically dispersed oil using a variable dilution design can provide a pragmatic strategy to support toxicity modeling. A further advantage of the variable dilution test design is that the toxicity test endpoint (e.g., LC_{50}, EC_{50}, EC_{10}) can be expressed in terms % WAF dilution. The toxic potency of the WAF stock can then be directly quantified by expressing toxicity test results in terms of toxic units (TU):

$$TU_{WAFstock} = 100/EffectEndpoint(\%\,dilution) \quad (1)$$

The utility of this approach compared to traditional concentration-based metrics for reporting toxicity results, is that use of equation (1) explicitly accounts for the bioavailability, concentration and composition of all mixture components in contributing to observed WAF toxicity. This strategy for aquatic hazard assessment has provided the technical basis for water quality-based control of complex whole effluents (USEPA, 1991 use of TU as an alternative exposure metric in evaluating oil toxicity tests is further detailed in Parkerton et al. 2023.

2.1.2. CEWAF method—Dispersants are products applied in oil spill response to encourage droplet formation and promote dispersion into the water to reduce slick oil exposures and associated impacts at the surface and shorelines. While increasing exposures in the water column, dispersant application increases oil surface area thereby promoting dissolution and biological degradation (NASEM, 2020). CROSERF developed a method for preparing media with oil+dispersants, referred to as chemical enhanced water accommodated fraction (CEWAF), to evaluate the hazard of chemically dispersed oil. The recommended media preparation method stipulated a 18–24 h mixing period, 20–25% vortex, 3–6 h settling period, and a 1:10 dispersant to oil ratio (DOR) (Aurand & Coelho, 2005). While specific guidance was not provided regarding the domain of oils amenable to CEWAF testing, light petroleum substances (e.g., gasoline), as well as heavy or highly weathered oils are not typically targeted in response efforts for dispersant treatment and thus are inappropriate for CEWAF tests. While fresh oils typically exhibit high dispersability, some weathering is likely before dispersants can be deployed in the field. As a result, CEWAF tests intended to reflect surface spill scenarios should rely on oils that are first weathered over a short period, e.g., 12 h yet remain dispersable. Depending on study objectives, weathering can be performed either artificially or naturally. Approaches for pre-weathering oils prior to testing and considerations for documenting procedures are discussed in Dettman et al. (2023). To address subsurface release and dispersant use scenarios where lower dispersant application rates may be effective (Brandvik, et al. 2013), CEWAF testing with fresh oils using lower dispersant to oil ratios (DORs) may be warranted.

It is recommended that future CEWAF testing focus only on oils that are found to be effectively dispersed using the dispersant investigated. In addition to the test oil, the nature and temperature of the test media selected for media preparation and used in subsequent toxicity testing must be considered since the effectiveness of chemical dispersants can be

strongly influenced by ionic strength and temperature (Fingas et al. 1991). A suggested criterion for documenting that test oil is effectively dispersed using the dispersant, DOR and test media selected is to demonstrate that oil concentrations in CEWAFs are five-fold higher than comparative WAFs consistent with the Tier II SMART protocol that is applied in evaluating dispersant effectiveness in the field (NOAA, 2006).

Test variables for WAF preparation identified in Table 2 are also relevant to CEWAF tests and require documentation. An additional consideration is how and when to add the dispersant to oil (Aurand & Coehlo, 2005). CROSERF recommended adding dispersant via a gas-tight syringe to the center of the WAF vortex following oil addition. Maximum oil loadings in CEWAF tests should be selected to reflect recommended field application rates and yield nominal dispersant concentrations that do not exceed the dispersant-only toxicity (i.e., LC50 in acute studies, EC10 in chronic tests). For example, if an oil loading of 1 g/L with a DOR of 1:10 is applied and the dispersant-only LC50 is 10 mg/L, one expects the dispersant could contribute significant toxicity in the resulting test media. In this illustration, a maximum oil loading of 0.1 g/L would be required. If a lower DOR of 1:100 is considered relevant, as is typical for subsea dispersant use (Mitchelmore et al. 2020a), a higher oil loading could be used. The requirement to limit dispersant exposures in CEWAF tests below concentrations expected to cause direct toxicity to the test species/endpoint being investigated provides another constraint on the selection of test oils that exhibit compatible toxicity. In the above example, an oil that is not toxic at WAF loadings below 1 g/L would not be amenable to CEWAF testing with this dispersant since oil loadings needed to cause oil toxicity would yield dispersant concentrations that are over an order of magnitude above the dispersant LC50. Moreover, if WAFs and CEWAF were prepared at 1 g/L for this oil with and without this dispersant using a 1:10 DOR and toxicity results compared, chemically dispersed oil would likely be deemed toxic due to dispersant while physically dispersed oil would be non-toxic. Based on these findings, one could erroneously conclude that dispersant increases oil toxicity when in fact the comparative toxicity of physically versus chemically dispersed oil is confounded by extreme dispersant concentrations as shown by Lee et al. (2013). Failure to properly document and design CEWAF tests can lead to unsupported conclusions that misguide decision-making (Coehlo et al. 2013). Adhering to the above recommendations for selecting appropriate test oils and maximum oil loadings in media preparation for CEWAF tests should provide toxicity studies that better inform spill response.

Since oil surface area is higher in CEWAF tests, mass transfer for oil dissolution is enhanced so that time required to achieve equilibrium of dissolved oil exposures is reduced when contrasted to physical mixing. However, empirically confirming equilibrium between neat and dissolved oil is challenging since test media includes both dissolved and droplet forms which are not differentiated using traditional analysis methods (Redman & Parkerton, 2015). A simple, conservative approach is to adopt the same mixing period and energy shown to provide equilibrium in a comparable WAF preparation without dispersant.

In contrast to WAFs, the objective of CEWAFs is to provide both dissolved and oil droplet exposures for toxicity evaluation. Oil droplet concentrations in the CEWAF stock will be dynamic and depend on the test oil, dispersant, DOR, test media, mixing energy, resulting

oil droplet size distribution, and selected settling time after mixing is terminated. Recent work by Forth et al. 2017b showed that oil droplets in CEWAFs exhibited a mean volume diameter ranging from 12–18 μm after 18–24 h of mixing prior to settling with 4 oils varying in extent of weathering (APIG from 36 to 9.7). After 24 and 100 h of settling, the mean volume diameter of oil droplets remained at around 5 μm. The depth of the water volume was not reported in this study to allow direct comparison to the estimates provided in Figure 4. However, using Figure 4 as a guide an additional 10 to >24 h would be required for 5 μm droplets to be removed from a 10 cm depth depending on the oil droplet density. Measured TPAH concentrations in droplet form for source and artificially evaporated oil CEWAFs were also reported to be more than an order of magnitude higher than observed for more weathered field collected slick oils tested. These findings appear consistent with visual differences observed in transparency for light and extra heavy oil CEWAFs following a 4 h settling period (Figure 2B).

While droplet oil exposures differ between WAF and CEWAFs, as illustrated in Figure 2, at a given oil loading dissolved oil exposures are expected to be comparable at equilibrium in accordance with Raoult's Law (Shiu et al. 1988; Nordtug & Hansen 2021). Raoult's Law states that at equilibrium the aqueous concentration of a liquid oil component is proportional to its mole fraction in the oil and the aqueous solubility of pure liquid component. Thus, using a variable loading approach as proposed by CROSERF and ensuring sufficient mixing time is provided to achieve equilibrium during WAF preparation, initial dissolved oil exposures derived from both oil (WAF) and oil+dispersant (CEWAF) preparation methods will be consistent. However, once WAF/CEWAF solutions corresponding to the different oil loading treatments are transferred to exposure vessels used for toxicity testing, dissolved oil exposure concentrations may be more effectively maintained in CEWAF media due to the presence of droplet oil that can dissolve and buffer potential losses. Greater stability of dissolved oil exposures has been documented in CEWAF versus WAF tests with Alaska North Slope crude oil based on passive sampling measurements at the end of static tests (McConville et al. 2018). A practical challenge can arise in conducting variable loading CEWAF tests if the test oil selected exhibits much higher toxicity than the dispersant. For example, if the test oil alone shows significant toxicity in WAFs at oil loadings of < 0.05 g/L, then the ability to reliably add dispersant at a DOR of 1:10 in a 2 L test vessel at lower oil loadings (e.g., less than ca. 5 μL dispersant) may become problematic. As a result, larger WAF test systems or pre-mixing of dispersant into oil may be required for performing CEWAF tests with more toxic oils.

When applying a variable dilution approach, the potential influence of droplet dissolution on dissolved oil exposures can be predicted using mass balance modeling as the droplets present in the CEWAF stock are diluted and must re-equilibrate (Parkerton et al. 2023). In order to re-establish equilibrium at each test dilution, oil components dissolve from oil droplets carried over from the CEWAF stock and thus act as a new source of oil to the aqueous test media. This contrasts with WAF stocks, in which oil droplets are often not present at high enough concentrations to appreciably alter dissolved oil exposures in treatment dilutions (Redman & Parkerton, 2015). Consequently, divergent dissolved oil exposure profiles can result when CEWAFs are prepared using variable dilution. Thus, it is impossible to differentiate the direct toxic effect of the dispersant and/or undissolved

droplets from the changes mediated by the dispersant on oil bioavailability and resulting dissolved oil exposures. This helps explain why CEWAFs appear more toxic than WAFs in variable dilution tests designs when the test oil is effectively chemically dispersed (NASEM, 2020). However, to empirically confirm the predicted influence of oil droplet dissolution in modulating dissolved oil exposures, conventional analytical methods are insufficient since reported concentrations do not differentiate dissolved from droplet forms. Instead, alternate methods, such as passive sampling measurements, are needed that quantify freely dissolved concentrations. Key concepts that dictate differences in oil exposures between variable loading and dilution designs are further illustrated and discussed by Mitchelmore et al. 2020a.

Guidance outlined above for oil+dispersant media preparation can be extended to other classes of spill response agents. Further, if CEWAF tests with other spill response agent tests are to be performed, the manufacturer-recommended product to oil ratios should typically be used to promote comparability between studies. However, depending on the study goal, a range of product-oil ratios may be investigated. For example, such toxicity test designs can be used to provide data to support the development of effect models that quantify the contribution of oil and spill response agent mixtures to observed effects (Parkerton et al. 2023). Media preparation using direct addition flow-through methods (see section 2.2) with or without spill response agents may also be considered since these methods can deliver more controlled exposures than traditional WAF/CEWAFs particularly for test oils containing more volatile constituents.

2.1.3. HEWAF method—Generation of high-energy water accommodated fraction (HEWAF) is achieved by mixing oil into the test media using an electric blender. This approach was implemented in USEPA's toxicity testing requirements for listing dispersants under the National Oil and Hazardous Substances Pollution Contingency Plan (USEPA, 1996). Under this rule, test media (synthetic seawater) were prepared by mixing a fuel oil with or without dispersant at a nominal loading of 1 g/L and DOR of 1:10 in a blender at speeds less than 10,000 rpm for 5 seconds. The blended stock solution was then diluted to compare oil toxicity in the presence and absence of dispersant. This approach was subsequently adopted by Incardona et al. (2013) to generate larger volumes of test media for oil toxicity testing. This approach has been subsequently applied generally without dispersants with some exceptions (Adams et al. 2014). HEWAFs were broadly adopted in toxicity studies related to the Deepwater Horizon (DWH) event in an attempt to mimic the high-energy environment associated with the blowout (Michelmore et al. 2020b). This method involved blending source or weathered DWH test oils with a Waring CB15 commercial food blender on the low setting (15,000 rpm) for 30 seconds. The blended WAF was then transferred to a separatory funnel and allowed to settle for 1 h with the resulting media used as soon as possible but no more than 24 h (Forth et al. 2017a).

Advantages of the HEWAF method include low oil use, short preparation times, and higher, initially stable, oil concentrations, providing good analytical reproducibility (Forth et al. 2017a). A further benefit is that high energy mixing can produce similar or smaller oil droplets than chemical dispersion, promoting more rapid equilibration between liquid and dissolved oil phases in the test media than physical mixing (Sandoval et al. 2017, Forth et al.

2017b). One limitation is characterization of test media using conventional GC-MS analysis indicates HEWAFs are less stable than WAF and CEWAFs over short settling times (<24 h) presumably due to coalescence of physically dispersed droplets and removal by buoyant forces that bring oil droplets to the surface (Sandoval et al. 2017). Another concern is that the energy dissipation rate introduced by high-speed blending is estimated to be orders of magnitude higher and of a shorter duration than is expected by breaking waves (Whittlesey et al. 2017). The consensus opinion provided in the NASEM (2020) report was that a key limitation of the HEWAF method was the unrealistically high mixing energy applied. As a result, it was recommended to avoid the use of HEWAFs unless there is conclusive field evidence to justify the resulting elevated microdroplet exposures associated with HEWAFs are environmentally relevant. However, if limited oil is available, HEWAF preparation may provide the only feasible media preparation option for static or static renewal tests. Further, providing dissolved and droplet oil exposures can be reliably quantified in such tests, resulting toxicity data can be valuable in evaluating toxicity models.

For assessing dissolved oil exposures, the same principles described for CEWAF apply to HEWAF. At a given oil loading, the dissolved oil composition and concentration at equilibrium will be equivalent across WAF, CEWAF, and HEWAF preparation methods, as shown by Forth et al. 2017a. In contrast, differences in total measured concentrations of oil constituents between WAF vs CEWAF or HEWAF reflect the added amount of oil that is introduced into the test media in droplet form due to chemical or high energy physical dispersion. For example, TPAH concentrations in WAF, CEWAF and HEWAF test media prepared at 1–2 g/L loading with artificially weathered source oil of Macondo source oil averaged 167, 3,685 and 6,836 μg/L, respectively. Thus, in this example chemical dispersion appears to increase TPAH in droplet form by 20-fold while high-speed blending further increases the droplet phase by another two-fold. As previously discussed, the amount of droplet oil will be influenced by the type of oil and the settling period chosen. Thus, if either CEWAF or HEWAF stocks are used to prepare test media using a variable dilution approach, oil droplets will be carried into the dilutions. As a result, the amount of oil introduced into each test dilution varies and the variable dilution approach becomes in effect a variable oil loading test. However, unless the total oil in the CEWAF or HEWAF stock is quantified, the actual oil loading at each test dilution is unknown.

The influence of high energy mixing on oil bioavailability can be quantified by applying equation (1) to variable dilution tests using HEWAF and WAF stocks prepared with the same oil and loading. This is illustrated in Table 4 using the two toxicity data sets for bay anchovy embryo' (O'Shaughnessy et al. 2018) and red drum larvae (Morris et al. 2018). The large discrepancy in the potency that is revealed by expressing observed toxicity of HEWAF vs WAF stocks in terms of TU highlights the extent to which droplet dissolution effectively enhances dissolved oil exposures. This insight is lost if TPAH-based effect concentrations are simply compared which differ within a factor of two between media preparation methods (Table 4). The utility of adopting $TU_{WAF\ stock}$ an exposure metric for consistent interpretation of variable dilution oil toxicity tests using WAFs and CEWAFs is further discussed in Parkerton et al. (2023).

In summary, in a variable dilution test, the dissolved oil concentration and composition at each dilution depends on amount of oil in the CEWAF/HEWAF stock since this dictates the oil loading in the dilutions. Since conventional analysis of oil exposures in test media does not differentiate dissolved from droplet forms, use of HEWAF/CEWAF media preparation coupled with a variable dilution approach complicates toxicity test interpretation since it is unclear how to causally link observed effects to dissolved or droplet oil exposures. If such data are to be used to support toxicity modeling, total oil concentrations in the CEWAF/HEWAF stock must be measured so that the oil loadings in dilutions are known. Additional steps to simulate or measure dissolved oil exposures in variable dilution tests are discussed in Parkerton et al. 2023.

An alternative strategy that could leverage advantages of HEWAF preparation while avoiding some shortcomings outlined would be to use a variable loading approach and adjust the settling time to better control droplet oil exposures. Previous work suggests that after a 24 h settling period, stable exposures containing < 10 μm oil droplets could be provided for up to 100 h using HEWAFs (Sandoval et al. 2017, Forth et al. 2017b). Figure 4 predicts that droplets of this size would exhibit longer rise times and yield potentially more stable droplet exposures for evaluation in toxicity tests. This strategy could help to effectively differentiate the role of dissolved (using WAFs) from droplet oil (using parallel HEWAFs) in contributing to observed effects assuming droplet concentrations and sizes tested are environmentally relevant.

2.1.4. Sheen oil method—Another direct addition method for media preparation applied following DWH is sheen oil toxicity tests (Morris et al. 2015; Mager et al. 2017). This method involves adding a known volume of test oil to the surface of exposure vessels that contain test organisms to achieve a thin oil layer. This media preparation method has been used to investigate the toxicity of oil sheens in the presence and absence of UV light (Key et al. 2020, DeLorenzo et al. 2021, Alloy et al. 2022). The amount of oil added to the surface can be varied to provide different oil layer thicknesses and resulting aqueous exposures. In the study by DeLorenzo et al. 2021, oil thicknesses of 0.25 to 4 μm were investigated in ca. 8 cm diameter crystallization dishes containing 200 mL of seawater and mud snails, grass shrimp, or sheepshead minnows. These oil thicknesses correspond to relatively low oil loadings on the order of 1–20 mg/L. A Teflon straw standpipe was incorporated into the exposure chamber to allow sampling of the water below the slick for oil analysis. Results indicated that after 1 d of equilibration, concentrations of TPAH50 in the water below the slick increased linearly with oil thickness.

Advantages of this approach include its simplicity and small volumes of oil and water required for testing. In addition, use of resulting test media focuses on the effects of the dissolved oil exposures that passively diffuse from the overlying slick limiting complications associated with possible effects of oil droplets. However, potential physical effects of test organism interactions with neat oil on the water surface can be assessed (Black et al. 2021). A key challenge with this procedure is that dosing occurs in an open test system so that concentrations in the sheen oil can change during the test due to volatilization. Differences in the dissolution kinetics of the oil components will also likely contribute to dynamic dissolved oil exposures that may not reflect equilibrium concentrations and be

homogenously distributed vertically through the exposure vessel and potentially modulated by induced mixing from test organism movements. Thus, multiple samples over depth and time during the test period may be required to accurately characterize dissolved oil exposures. Oil sheens may also serve as a barrier for oxygen exchange and cause unacceptable water quality conditions, potentially confounding test results. Additional research is needed to evaluate the utility of sheen oil toxicity tests for hazard assessment and toxicity model validation and opportunities for further standardization, including obtaining and documenting acceptable water quality.

2.1.5. Large volume media preparation test systems—Standard toxicity tests typically employ small test species or the early life-stages of larger organisms and typically require test media volumes of <100 mL up to 1 L per replicate. Large volume, open experimental systems (Dussauze et al. 2015) and outdoor wave tanks (Greer et al. 2012) have been used for media preparation and can be used for subsequent toxicity testing with larger organisms such as adult fish. A novel approach has been described that employs a constructed 39,000 L inland recirculating oil exposure facility (Main et al. 2018). This system was designed to maintain water quality during pulsed, spiked, and constant, short-and long-term oil exposures studies. Test solutions were made following CROSERF protocols for WAF or CEWAF exposures with some adaptations required for scaling. Due to the large volumes of solution needed for the exposure tanks (9–500 L coated with an isophthalic acid gelcoat to prevent adsorption of hydrocarbons), 25 L carboys and oversized magnetic stir plates were used to make stock solutions daily. Dilution of the stock solutions to generate the large volume of solutions with the targeted TPH or TPAH concentration needed was carried out in 3–1400 L isophthalic acid-coated mixing reservoirs equipped with a rotating propeller. Diluted stocks were mixed for 30 minutes before cycling through exposure tanks. Analytical confirmation of TPH and TPAH concentrations was carried out daily on stock, mixing, and exposure tank solutions to evaluate the stability of oil exposures. Key challenges with large volume systems include practicality, lack of standardization, and characterization of the dynamic dissolved and droplet oil exposures that result so that test exposures can be linked to observed and predicted effects. Additional methods used to introduce oil into mesocosms of varying scales are reviewed by Wade et al. (2022).

2.2. Direct addition / flow-through methods

CROSERF provided an early option for conducting closed, once flow-through exposure tests (i.e., spiked declining exposure). In this test, WAFs, CEWAFs, or dispersants alone are first prepared. The dosed test media is then placed in flow-through test chambers to which test organisms are added. Pumps are then turned on so that the 240 mL volume test chambers are diluted with clean water using a flow rate of 2 mL per minute (Aurand & Coelho, 2005). This rate provides a hydraulic retention time of 2 hours and an estimated concentration decline half-life of ca. 1.67 h in test chambers with tests typically carried out for 48 to 96 h. This dilution rate was selected to mimic the observed rapid decline of oil concentrations below slicks during field spills (Singer et al. 1991). Field trials at sea under open ocean conditions have shown that high concentrations of physically or chemically dispersed oil in the top meters of the water column following initial spiking decline within minutes to hours (≤4 h) to concentrations of ≤ 1 mg/L (Bejarano et al. 2014).

For WAFs investigated in this test system, conventional analytical measurements can confirm the modeled decline in dissolved oil exposures. However, for spiked CEWAF tests, the combined role of dilution and droplet dissolution processes results in uncertain dissolved oil exposures. Thus, to validate time variable effect models for oil or dispersant, spiked declining exposures with WAFs or dispersant alone have greater utility (French-McCay et al. 2023). However, limited spiked toxicity studies available for dispersant or WAFs ((Singer et al. 1991; Clark et al. 2001; Van Scoy et al. 2012) have principally been used to demonstrate the degree of conservatism provided by traditional constant exposure toxicity tests rather than to support toxicity modeling. The influence of exposure duration on observed oil toxicity in lab and wave tank studies can also be used to quantify differences in effects between continuous and pulsed exposure scenarios (Greer et al. 2012; Stubblefield et al. 2023).

An alterantive to the CROSERF approach is the continuous oil droplet generator system described by Nordtug et al. (2011a) and depicted in Figure 5. Oil (or oil+dispersant) and clean seawater are pumped into a droplet generator with multiple compartments connected by 0.5 mm nozzles. As a result of the turbulence produced, a defined, stable dispersed oil concentration and droplet distribution can be produced. This system has been used to generate “stock solutions” of dispersed oil in the range 0.2 to 50 mg/L with median volumetric oil droplet sizes in the range 10 to 30 μm for use in toxicity and biodegradation studies. Dilutions are made by adding various amounts of water to the continuously produced “stock” dispersion. At each dilution step, new equilibria between oil and water are established and the dilution series should thus be regarded as a “variable loading” test design. The resulting “stock” eluent from the droplet generator is further diluted with clean seawater to provide different nominal oil concentrations ranging from 0.2 to 50 mg/L. For each dilution, the resulting flow can be further divided into two equal streams so that one stream can be passed through a filtration system to remove oil droplets. The filtration system consists of loosely packed fine glass wool on top of stacked Whatman glass fiber filters that retain 1.6 and 0.7 μm sized particles. Time variable exposures can also be readily achieved by varying the addition of oil without changing water flow. Since exposure chambers are not sealed, this is considered an open test system.

This dosing system has been applied in multiple studies to investigate the toxicity of unfiltered and filtered, dispersed oil exposures with and without dispersant (Table 1) as well as bioconcentration of oil components (Øverjordet et al. 2018; Hansen et al. 2018b). An advantage of this system is that relatively large volumes of test media can be generated (250 L/d) with a limited amount of test oil consumption (a few mLs of oil per day). Droplet size distributions can be adjusted by the water flow rate into the generator but is normally limited to the median droplet size range between 10 to 30 μm (Brakstad et. al. 2015), thus covering the smaller droplet fraction expected to be generated during oil spills. A limitation is that if differences in toxicity between unfiltered and filtered treatments are observed, several explanations are possible. Additional toxicity of unfiltered treatments may be mediated directly by droplets, as previously discussed, or as an indirect result of modulating dissolved oil exposures (Hansen et al. 2019a). To further elucidate the role of dissolved and droplet oil, more detailed characterization of dissolved oil exposures in unfiltered and filtered treatments are thus required using either exposure modeling (Hansen et al. 2019b) or

analytical methods that can differentiate dissolved from total oil concentrations (Dettman et al. 2023). As previously mentioned, oil droplet exposures generated for lab toxicity studies should be informed by measured or predicted droplet concentrations, compositions, sizes, and exposure durations characteristic of field spill scenarios.

2.3. Indirect addition

2.3.1. Static or static renewal methods—Passive dosing (PD) techniques are an important advance in aquatic toxicity testing (Smith et al. 2010). The general principle involves equilibrating a test substance (neat or dissolved in a solvent such as methanol) with a polymer and then introducing "loaded" polymer into the aqueous test media to provide rapid, partition-controlled delivery of the test substance. This approach represents the reverse process upon which passive sampling methods used for reliable, sensitive analysis of bioavailable exposures of hydrophobic chemicals are based (Vrana et al. 2015). Silicone, typically in the form of polydimethylsiloxane (PDMS), has commonly been employed due to its low cost, wide availability, high sorptive capacity, and favorable sorption/desorption kinetics (Rusina et al. 2007). PDMS is available from different suppliers but has been shown to have identical absorption capacities than other classes of silicones (Gilbert et al. 2016). This polymer has been used in multiple formats for PD, including coated vials (Brown et al. 2001; Kiparissis et al. 2003; Turcotte et al. 2011; Lin et al. 2015), O-rings (Smith et al. 2010; Bragin et al. 2016; Niehus et al. 2018; Bera et al. 2018; Stibany et al. 2020; Butler et al. 2020; Philbert et al. 2021; Colvin et al. 2021), tubing (Kang et al. 2014; Redman et al. 2017) and rods (Trac et al. 2018; Hammershøj et al. 2020; Trac et al. 2021).

PD systems are typically designed so that the mass of substance loaded in the PDMS donor is much greater than the mass at equilibrium in the test media plus any losses occurring over the exposure period. When these negligible depletion conditions are met, the PDMS concentration will not be appreciably reduced, and the corresponding dissolved concentration in the test media will be maintained. Further, dissolved concentrations can be simply calculated from the test substance concentration in PDMS divided by the substance's PDMS-water partition coefficient. Most PD studies relevant to oil spill contexts have focused on evaluating toxicity of single oil components or defined synthetic hydrocarbon mixtures (Rojo-Nieto et al. 2012; Niehus et al. 2018; Philibert et al. 2021). A key advantage of PD is the capability to provide well-controlled exposures for hydrocarbons that are prone to loss in aquatic toxicity tests due to sorption, volatilization, and abiotic/biotic degradation. Studies have shown that traditional spiking methods can significantly understate the toxicity of such substances due to declining exposures, while PD methods yield more reliable effects data indicating higher toxicity (Smith et al. 2005; Rojo-Nieto et al. 2012). A further benefit of PD is that test substance exposures are constrained to the test substance's solubility limit so the potential physical effects of undissolved test substances do not confound toxicity test interpretation (Seiler et al. 2014; Stibany et al. 2020). Thus, PD enables generation of more accurate toxicity test data that can serve as critical input to quantitative effect model development and calibration (French-McCay, 2023). PD also provides opportunity to investigate the potential role of dissolved and dietary exposures in contributing to toxicity (Fisher et al. 2016).

Recently, PD techniques have been extended to crude oil and petroleum substances (Table 5). Several preliminary conclusions can be reached based on these studies. First, the time required to reach equilibrium between test media and passive donor is on the time scale of hours, with silicone cord exhibiting the fastest reported kinetics. Second, all three PD methods provide dissolved oil exposures that appear comparable to low-energy WAFs. Third, silicone O-rings/cords are practically simpler to apply than the preparation of oil-filled tubing. Fourth, O-ring and silicone cords absorb oil within 24 h with lighter oils causing more swelling (i.e., absorb more mass) than heavier oils. Lastly, the sorptive capacity of silicone cords appears higher than O-rings, likely due to the presence of iron oxide fillers in O-rings. PD methods for oil toxicity testing appear to be best suited for test oils where losses in WAF static/static-renewal tests are unacceptable or in cases where longer-term chronic exposures are required. This media preparation method may also provide an improved basis for comparing the toxicity of dissolved oil exposures to dissolved+droplet oil exposures obtained using CEWAF/ HEWAFs prepared at equivalent oil loadings.

A second indirect approach for preparing test media involves equilibration with test substance in overlying air or bubbling air saturated with test substance directly into the test media. This approach is particularly suited for toxicity testing of volatile aliphatic hydrocarbons (Trac et al. 2019; Parkerton et al. 2020). However, this method is generally less amenable to oil and petroleum substances which typically contain non-volatile components that do not readily partition to the headspace (Trac et al. 2021).

2.3.2. Flow-through methods—Several methods for preparing test media involve either once-through or recirculating test systems. An earlier method involved soaking test oil into an inert sorbent (chromasorb) followed by packing this material into a generator column through which clean water was pumped. The resulting eluent exhibited a composition that reflected the variation in the effective oil mass to water volume over time. Observed oil exposure profiles showed enrichment in more soluble hydrocarbons during early sampling times and less soluble oil components during later sampling periods consistent with model predictions (Shiu et al. 1988). This approach has been applied to assess the aquatic hazard of hydrocarbon resins (Woods et al. 2007).

A similar strategy that has been more broadly applied is use of generator columns packed with oiled gravel, sand or ceramic beads (Adams et al. 2017) with examples given in Table 1. To provide various treatments with a gradient of oil exposures for assessing dose-response relationships, different amounts of oil are applied to column media. This method was originally developed in an attempt to mimic chronic oil exposures associated with spilled oil that is transported to environments that provide critical habitat for fish spawning, embryo-larval development, and subsequent population recruitment (Marty et al. 1997). Consistent with the exposure pattern described by Shiu et al. (1988), exposure concentrations generated from these systems are expected to decline and change in composition over time as the source oil becomes progressively depleted due to component dissolution into the flowing aqueous media that is pumped through the column. Hossain et al. (2017) and Adams et al. (2021) modified this method so that water flowing through gravel packed columns mobilized oil droplets trapped in the wetted gravel substrate. This dosing approach was developed

to mimic hyporheic flows of water through river sediments where trapped oil could serve as a source of hydrocarbons to incubating salmonids. Another important aspect of these systems is that mass transfer constraints may preclude equilibrium with test media and limit dissolved oil exposures of less soluble oil components when contrasted to WAFs prepared at the same oil loading (Jung et al. 2015b; Hossain et al. 2017). Further, since oil is coated rather than absorbed to the solids packed into the column, undissolved oil released into the eluent complicates interpretation of analytical data since measured concentrations may reflect dynamic exposures of both dissolved and particulate forms of oil. The use of continuous once-through systems that rely on oil-loaded silicone discussed above, rather than oil-contaminated gravel or sand, may provide more reproducible dynamic dissolved oil that is amenable to time-dependent bioconcentration and toxicity modeling (Matthew et al. 2008). An elegant flow-through passive dosing system has been reported by Wang et al. 2022 that can generate large volumes of test media with stable aqueous exposures. This system uses hollow tubes that contain silicone rods that are contaminated with the test substance. This system was shown to be applicable to both individual hydrocarbons (fluoranthene) as well as a complex petroleum substance (gas oil).

Silicone-based PD techniques have been successfully incorporated into recirculating flow through systems to provide constant exposures for evaluating toxicity of hydrocarbons (Butler et al. 2016; Renegar et al. 2017; Turner et al. 2021) which has been identified as a priority research need for advancing toxicity models (French-McCay et al. 2023). A flow-through passive dosing (FTPD) system can also be designed to provide well-controlled time variable exposures of individual hydrocarbons or simple hydrocarbon mixtures. As previously mentioned, such systems are typically designed such that losses due to dissolution, evaporation and degradation result in negligible depletion of the test substance from the silicone donor so that stable exposure concentrations can be maintained. An example FTPD system that may be used to investigate dynamic exposures in future toxicity studies is detailed in Appendix S2. An accompanying spreadsheet that can guide the design and predict time variable exposures is also provided in Appendix S3. Further, if single PAH compounds are investigated using this approach, rapid and inexpensive fluorometric methods may be used to confirm exposure profiles over time.

3. Decontamination and waste disposal

Numerous protocols exist that describe how to prepare solutions containing crude oils, petroleum substances and/or chemical dispersants but rarely are details provided on how reusable equipment (i.e., glass aspirator bottles) should be cleaned and decontaminated or how to dispose of associated wastes. While hazardous waste management procedures vary significantly across local, national and global jurisdictions, examples of generic or specific approaches detailed in the literature are briefly provided below to highlight procedures that have been used for waste management. Before conducting any tests, protocols should be developed to ensure that waste disposal procedures follow applicable regulatory requirements. Waste is generated during exposure media preparation where consumables/equipment are used depending on the specific preparation used (e.g., glass syringes, metal spatulas and weigh boats, aspirator bottles, blenders, and separation funnels). Following media preparation, un-decanted/excess solutions remain that must be disposed. Liquid waste

is also generated during test exposures, the amount depending on the type of exposure system used (i.e., static renewal or flow-through systems) and may vary from mL (for static exposures with small organisms) to L quantities per day if working with larger batch or flow-through systems with larger organisms. During the testing period, exposure solutions may be sampled for chemical analyses. Contaminated equipment amenable to re-use needs to be cleaned (decontaminated for reuse) or disposed of after use, as do the solutions left over after and during analytical assessments. Finally, after testing, oiled equipment (e.g., beakers, test chambers) needs to be appropriately cleaned and re-used or disposed.

While specific protocols used for the disposal of solid/liquid oil and/or dispersant waste varies with type of oil, volume and nature of media and applicable regulations, usually waste (solid and liquid) is placed in approved containers, and commercial vendors who specialize in hazardous waste disposal will collect and properly dispose of the material. There are a number of protocols for decontaminating and reusing equipment (i.e., glassware, spatulas). Solid oil coating surfaces can be removed with appropriate wipes (e.g., paper towel wetted with hexane) that are then disposed of as solid hazardous waste. Glassware and other equipment are then further cleaned, which generally involves the use of a degreaser and/or detergent (with oiled waste solutions being disposed of as described for oil/water waste) followed with rinsing in distilled water followed by two or more solvent rinses (e.g., acetone, hexane, dichloromethane) or use of a muffle furnace. Baking glassware at high temperatures removes residual contaminants, although it is not suitable for all glassware, e.g., filtration glass frit apparatus.

The specific disposal technique used mainly depends on the estimated volume of waste generated throughout the experiment. Dedicated waste treatment systems are often used in large-scale experiments involving high oil-contaminated media volumes or prolonged chronic exposures. For example, a filtration and separation system described by Main et al. (2018) was employed. This system included a series of filters, and storage tanks to remove oil (and chemical dispersant). Analytical monitoring at various stages in the filtration/treatment processes demonstrated successful removal of oil contaminants by the filtration system and presence in the transfer/storage tank which was disposed of according to State hazardous waste guidelines (Main et al. 2018). Simpler approaches for the collection and disposal of oil waste involve use of an evaporative basin system. In this approach, a heavy non-tear plastic sheet is placed in a suitable container (e.g., 10 × 20 ft) so that liquid oil waste can be poured into this lined container system. The wastewater evaporates, leaving the oil residue behind. The liner is removed and disposed of as hazardous material.

Based on the experience of some authors, an activated charcoal filter has been applied for treating routine volumes of oil contaminated media generated from toxicity tests. In this system, exposure solutions are poured through an activated charcoal bed, and the resulting solution is captured and analyzed for oil residuals. If none are detected, the liquid waste can be sent to the sewage (WWTP) system. This system needs active monitoring, analytical verification before disposal, and regular changing of the charcoal filtration system. Once the charcoal has been used, it must be disposed as hazardous waste. For smaller volumes (e.g., invertebrate static or static-renewal testing), sending oil contaminated solutions directly to hazardous liquid disposal is often the easiest and most cost-effective method. Throughout

the test duration, exposure solutions (i.e., left over from preparation or following exposure renewals) can be directly decanted into glass 4L bottles or larger drum containers and directly sent for hazardous liquid disposal using a commercial vendor.

Summary & recommendations

Media preparation is a crucial aspect of oil spill-related aquatic hazard assessments. If resulting toxicity data are to be used to inform consistent hazard evaluation and support toxicity modeling the influence of the media preparation method on dissolved and droplet oil exposures and toxicity test outcomes must be understood. Multiple methods have been used to prepare physically and chemically dispersed oils depending on how oil is directly or indirectly added to the test media and if a static/static renewal or flow-through dosing regime is adopted. A variety of direct addition methods for spill response agents have been reported but the impact on toxicity test results have received little attention. Further research is needed to evaluate and further standardize media preparation for these products.

The direct addition, static/static renewal method originally proposed by CROSERF that relies on WAF/CEWAF generation is the most common media preparation method for oil toxicity testing. However, this method has been subjected to multiple modifications that can influence oil exposures, toxicity results, test interpretation and utility for effect model calibration/validation. Given this method will likely continue to find broad application, recommendations are provided for future application of this approach as well general guidance in selecting alternative protocols.

WAF preparation:

- Consider use of partially weathered test oil to reduce practical challenges associated with loss of volatile oil components.
- Apply more energetic mixing conditions for WAF preparation that is consistent with original CROSEF guidance for CEWAF preparation (i. e., 20–25 % vortex) to promote higher, more consistent dissolved oil exposures that better reflect equilibrium.
- Conduct a preliminary study prior to toxicity testing to tailor the WAF protocol to the oil and lab test conditions so that stable dissolved oil exposures can be provided.
- Select a maximum oil loading that is sufficiently toxic for establishing concentration-response relationships for the test organism/endpoint of interest while limiting the amount of test oil required and resulting waste.
- Document the basis and details of the media preparation protocol used in oil toxicity testing as this information is critical to evaluating study reliability (Bejarano et al. 2023).

CEWAF preparation:

- Select test oils with sufficient toxicity and dispersability with the dispersant being investigated. Avoid heavy or weathered oils that exhibit poor dispersibility

and/or low toxicity that can mischaracterize the comparative aquatic hazard of chemically versus physically dispersed oils.

- Investigate product to oil ratios that reflect recommended field applications rates.
- Avoid CEWAF treatments that yield nominal concentrations of the spill response agent which may cause direct toxicity from the agent alone.

Select Protocols Which Support Study Objectives:

- Understand advantages and limitations of variable loading versus dilution test designs. If the study aim is to evaluate toxicity of a defined dissolved oil profile, variable dilution can be used with potential analytical cost savings. However, if the objective is to compare the toxicity of a physically versus chemically dispersed oil, the variable loading design provides more comparable dissolved oil exposures upon which to evaluate the role droplet exposures contribute to toxicity.
- Consider use of HEWAFs prepared at variable oil loadings and adjustments to the settling time so that advantages of high energy mixing media preparation can be leveraged.
- Ensure droplet concentrations, compositions, sizes, and exposure durations are quantified and characteristic of field spill exposures for studies that aim to elucidate potential direct effects of droplet oil.

Alternative media preparation approaches discussed in this review have allowed the original scope of CROSERF that focused on short term exposures to small pelagic organisms to be expanded to address other spill exposure scenarios. This includes consideration of subsea releases in which volatile aromatic and aliphatic oil components can serve as a more important contributors to toxicity than in surface spills and response measures can differ both in the potential nature and application rate of spill response agents. Methods are also discussed to address potentially longer-term spill exposures that may occur during a well blowout or following oil contamination of natural substrates that may serve as a source of chronic oil contamination to the local aquatic environment. Approaches for generating the greater volumes of oil contaminated media that are needed for testing larger commercial test species than initially envisioned by CROSERF are also presented. Further, media preparation methods are described to support development of toxicity test data for not only test oils but also on individual compounds that are needed for advancing oil spill effect models. Recommendations for media preparation approaches that extend CROSERF guidance include:

- Evaluate the utility and potential standardization of sheen oil toxicity tests
- Apply passive dosing techniques for toxicity assessment of individual hydrocarbons to provide reliable effects data required for toxicity modeling
- Utilize direct oil injection or passive dosing flow-through systems if large volumes or longer test exposures of contaminated oil media are required for toxicity testing,

- Perform interlaboratory comparison to advance best practices and guideline development for passive dosing of crude oil and related petroleum substances

It has previously been suggested that future research in this arena would be best served via continued collaborative engagement between industry, government and academia (Hodson et al. 2019). We concur that this is a logical path forward for addressing the recommendations highlighted above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Multi-Partner Research Initiative under Canada's Oceans Protection Plan through Fisheries and Oceans Canada. We also would like to acknowledge Jose Luis (Pepe) Rodriguez for assistance in preparation of figures. This is contribution number 6289 and Ref. No. [UMCES] CBL 2023-059 of the University of Maryland Center for Environmental Science.

Data availability

The data included in this paper were obtained from published work or was generated by model simulations that are described in the text; a spreadsheet is included in SI for guiding preparation of controlled, time variable test substance exposures in aqueous media for use in future aquatic toxicity studies.

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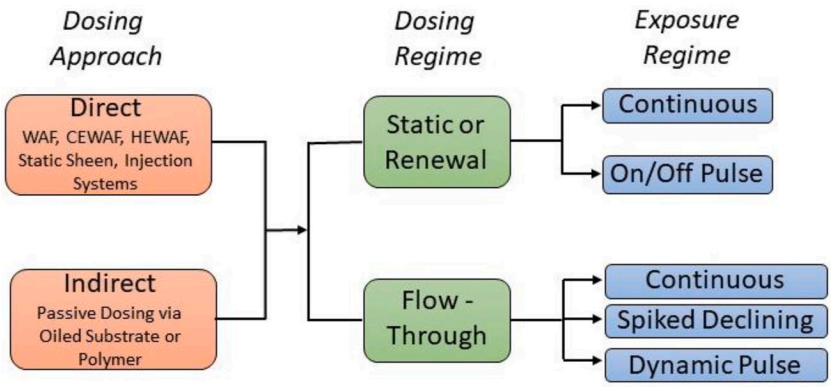

Fig. 1.
Overview of methods used for aqueous test media preparation

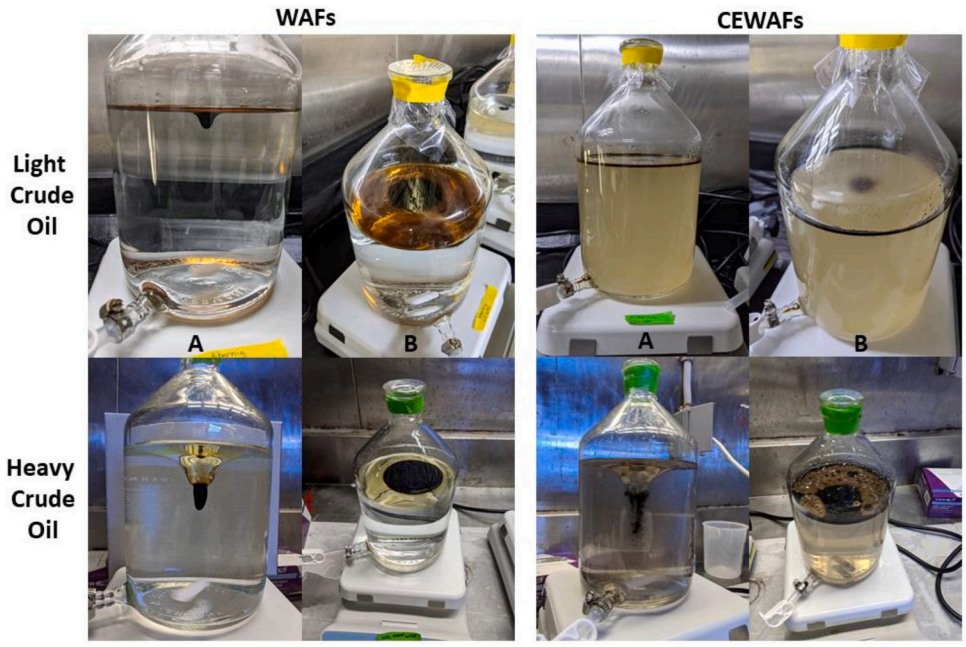

Fig. 2.

Comparison of physically (WAFs) and chemically (CEWAFs) dispersed oils during (A) mixing and (B) settling after 4 hrs. Note the different behavior of floating oil and increased droplet concentration in CEWAFs than WAFs for both oils.

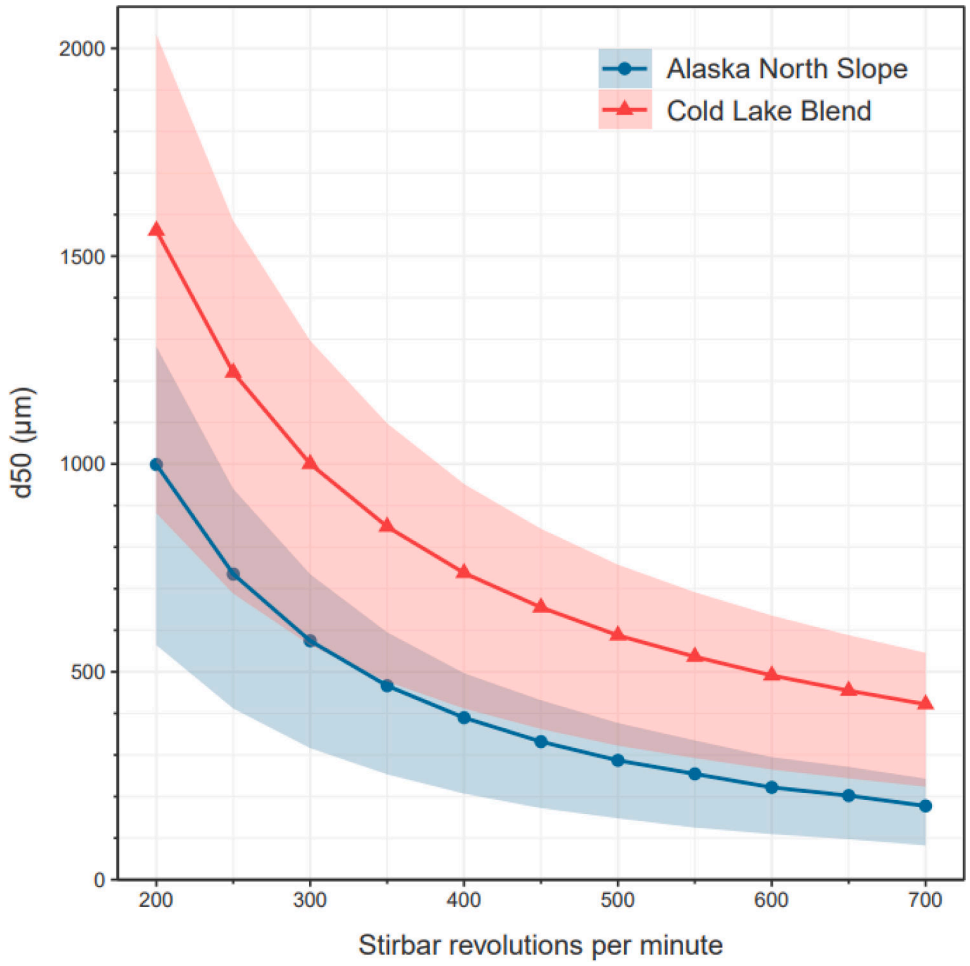

Fig. 3.

VDROP simulations showing influence of mixing speed on median oil droplet size for two oils in 4 L aspirator bottle with a 3.8 cm length stir bar during WAF preparation. Solid lines denote the median droplet size (d50) while lower and upper shaded area show the predicted droplet sizes corresponding to 10^{th} (d_{10}) and 90^{th} (d_{90}) percentiles of the droplet distributions.

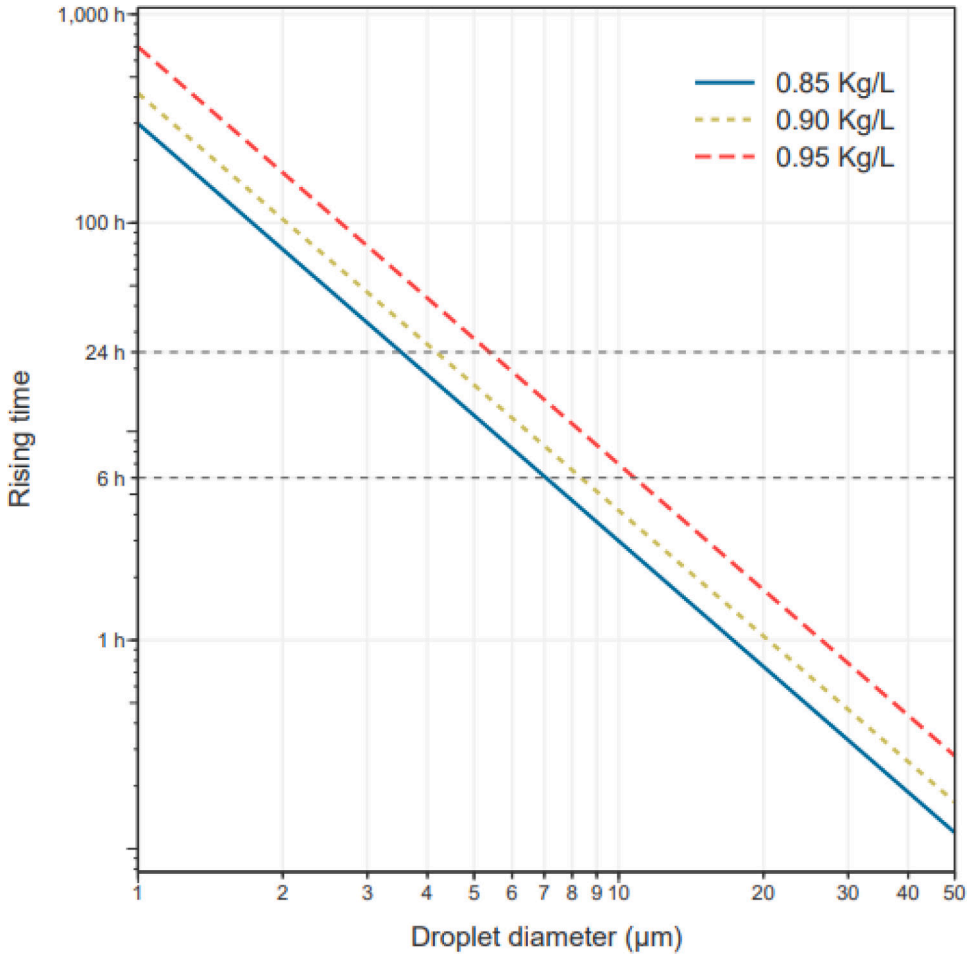

Fig. 4.

The rising time for spherical droplets to travel 10 cm in the water column due to buoyancy based on Stokes Law. Different lines highlight the influence of oil density on droplet rise times.

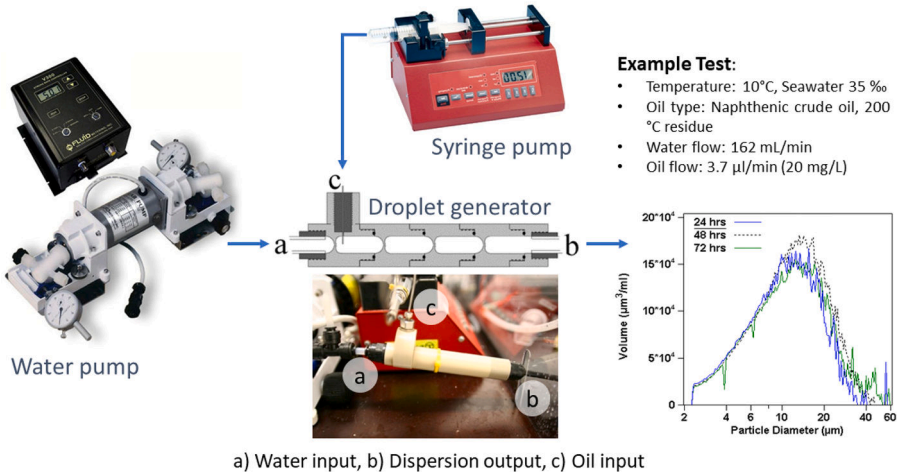

Fig. 5.

Schematic of the continuous oil dosing system described by Nordtug et al. (2011).

Table 1

Examples of studies that illustrate the use of different media preparation methods

Test Substance(s)	Dosing Approach	Dosing Regime	Exposure Regime	Example Citations
Dispersants	Direct	Static Open or Closed	Continuous Open	Scarlett et al. 2005; Hemmer et al. 2011; Hansen et al. 2014; Negri et al. 2018; DeLorenzo et al. 2018; Echols et al. 2019; Barron et al. 2020
Dispersants	Direct	Renewal Open	Continuous Open	Barron et al. 2020
Hydrocarbons	Direct	Static Closed	Continuous Open	Olsen et al. 2011; Geier et al. 2018
Hydrocarbons/ Dispersant	Direct	Static Closed	Continuous Closed	McConville et al. 2018
Oil, Oil+Dispersant	Direct	Static Closed	Continuous Open	Hemmer et al. 2011; Incardona et al. 2013; Steffanssson et al. 2016; Mager et al. 2017; DeLorenzo et al. 2018; Luter et al. 2019; Donohoe et al. 2021
Oil, Oil+Dispersant	Direct	Static Closed	Continuous Closed	Hook & Osborn, 2012; Negri et al. 2016; Faksness et al. 2020; DeMiguel-Jimenez et al. 2021
Oil	Direct	Static Open	Continuous Open	Klinger et al. 2015; Frantzen et al. 2015
Oil, Oil+Dispersant	Direct	Renewal Closed	Continuous Open	Couillard et al. 2005; Schein et al. 2009; Martin et al. 2014; Echols et al. 2016a; Barron et al. 2020; Hansen et al. 2021
Oil, Oil+Dispersant	Direct	Renewal Closed	Continuous Closed	Echols et al. 2016a,b, c,
Oil, Oil+Dispersant	Direct	Static Closed	On-Off Pulse Open	Gardiner et al. 2013
Oil	Direct	Static Open	Continuous Open	Greer et al. 2012; Dussauze et al. 2015; DeLorenzo et al. 2018;2021Key et al. 2020; Hansen et al. 2021
Oils	Direct	Once-through flow Closed	Spiked Declining Closed	Singer et al. 1991; Van Scoy et al. 2012
Oils	Direct Injection	Once-through flow Closed	Continuous Open	Nordtug et al. 2011a, Nordtug et al. 2011b, 2015; Hansen et al. 2012, 2015, 2017, 2018a, 2019; Olsen et al. 2013; Sørhus et al. 2015
Oils	Direct Injection	Once-through flow Closed	On-Off Pulse Open	Laurel et al. 2019; Olsvik et al. 2021
Hydrocarbons/Oil	Indirect Silicone	Static Closed	Continuous Closed	Turcotte et al. 2011; Rojo-Nieto et al 2012; Bragin et al. 2016; Redman et al. 2017; Stibany et al. 2017; 2020; Hammershøj et al. 2020; Butler et al. 2020; Philbert et al. 2021; Trac et al. 2021
Hydrocarbons/Oil	Indirect Saturated air	Static Closed	Continuous Closed	Trac et al. 2018,2019,2021; Parkerton et al. 2020
Hydrocarbons/Oil	Indirect Chromosorb	Once-through flow Closed	NA	Billington et al. 1988; Shiu et al. 1988
Oils	Indirect Gravel	Once-through flow Closed	Continuous Open	Martin et al. 2014; incardona et al. 2015; Jung et al. 2015a; Nahrgang et al. 2016; Holth et al. 2017;
Oils	Indirect Gravel	Once-through flow Closed	On-Off Pulse Open	Duan et al. 2018; Incardona et al. 2021
Oils	Indirect Ceramic Beads	Once-through flow Closed	Continuous Open	Kennedy & Farrell 2006; Alderman et al. 2017
Oils	Indirect Ceramic Beads	Once-through flow Closed	On-Off Pulse Open	Alderman et al. 2018; Avery et al. 2020; Alderman et al. 2020

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Test Substance(s)	Dosing Approach	Dosing Regime	Exposure Regime	Example Citations
Hydrocarbons	Indirect Silicone	Recirculating Closed	Continuous Closed	Butler et al. 2016; Knap et al. 2017; Renegar et al 2017; Renegar et al 2021; Turner et al. 2021

NA = not applicable as study did not include toxicity test exposures

Table 2

Key test variables and protocol objectives for WAF preparation

Variable	Objective
Maximum Oil Loading	Generate aqueous media with sufficient dissolved oil exposure to invoke a significant toxic response and to facilitate equilibrium
Mixing Energy and Duration	Achieve equilibrium between oil and aqueous test media within a short period (e.g., 24–48 h) while avoiding entrainment of oil droplets that cannot be readily removed via settling
Settling Period	Limit the amount of droplet oil in WAF so that traditional hydrocarbon analyses represent dissolved oil components
Mixing Chamber	Limit loss of volatile components by using a closed vessel
Lighting Regime	Prevent light exposure and potential photo-oxidation by mixing in the dark or covering the vessel
Variable Loading Design	Simulate changes in dissolved oil composition in treatments as a function of oil loading
Variable Dilution Design	Maintain constant dissolved oil composition in treatments as a function of stock WAF dilution

Table 3

Screening study for defining a WAF protocol.

Variable	Description
Mixing Vessel	1 to 20 L sealed glass aspirator bottle with 80% test media, 20% headspace, and 3 to 16 cm stir bar depending on vessel volume
Oil Loadings	One or more loadings, e.g., 0.1, 1 g oil /L control media are selected. WAFs generated using these loadings can also be used in analytical screening and range-finding toxicity tests to ensure stable exposures and high enough concentrations to elicit toxicity for the organism/endpoint being investigated
Mixing Energy	Moderate (20–25% vortex) physical mixing is employed using magnetic stir plates to promote equilibrium between oil and test media with minimal droplet formation. Higher mixing energies are avoided to decrease the probability of generating small oil droplets that are less effectively removed during the settling period
Mixing Duration	Different mixing times, e.g., 8, 24, 48, 96 h are selected to assess evidence of equilibration
Equilibrium determination	Measure 280 nm excitation/340 nm emission (reflecting 1–3 ring PAHs) and 280 nm excitation/445 nm emission (reflecting > 3+ PAHs) intensities in WAF over time (Kepkay et al. 2008). Alternatively, other methods could also be used, such as GC-FID peak areas or total organic carbon measurements based on method detection limit and cost considerations (Girling et al. 1994; Ali et al. 1995; OECD 2019)
Settling Period	Once mixing time to achieve WAF stability is determined, investigate different settling times to characterize droplet exposures e.g., 2, 6, 24 h
Stability Determination	Measure ratio of 280 nm excitation/340 nm emission (1–3 ring PAHs) and 280 nm excitation/445 nm emission (> 3+ PAHs) intensities for different settling periods. Test media can also be analyzed for total saturated hydrocarbons to evaluate evidence of droplets

Table 4

Comparison of toxic units[1] in stock WAF[2] and HEWAF using a variable dilution approach and $TPAH_{50}$ as the concentration-based exposure metric.

Study	Organism	Exposure Duration/ Endpoint	Oil	WAF method	Exposure Metric	WAF stock concentration (ug/L)	Effect Concentration (ug/L)	$TU_{WAF\ stock}$
O'Shaughnessy et al. 2018	bay anchovy embryos	48 h mortality	Slick A	WAF	TPAH50	10	4.3	2.3
				HEWAF		2373	3.9	608.5
			Slick B	WAF		4	1.6	2.5
				HEWAF		263	1.5	175.3
Morris et al. 2018	red drum Larvae	36 h periocardial area	Slick A	WAF	TPAH50	18	4	4.5
				HEWAF		1575	17	92.6
			Slick B	WAF		15	9	1.7
				HEWAF		345	17.5	19.7
		36 h ventricular contractility	Slick A	WAF	TPAH50	18	2	9.0
				HEWAF		1575	4.5	350.0
			Slick B	WAF		15	2.5	6.0
				HEWAF		345	3.5	98.7

Study A = O'Shaughnessy et al. 2017; Study B = Morris et al. 2018;

[1] calculated using equation 1;

[2] prepared using low energy mixing (no vortex)

Table 5

Summary of Studies Using Passive Dosing Methods to Prepare Test Media with Crude Oils and Petroleum Substances

PD Donor	Silicone tubing	Silicone O-rings	Silicone Cord
Loading Donor	Pump test oil into the tube and tie a knot at the end	Soak O-rings in liquid test oil	Allow cord to absorb a known amount of test oil
Generating Treatments i.e., Varying Oil Loading	Vary lengths of oil filled tubing in a fixed volume of media	Vary number of O-rings with a known sorbed mass of oil in a fixed volume of media	Vary the amount of oil absorbed to the cord at fractions up to sorptive capacity in a fixed volume of test media
Oils Tested	Iranian heavy crude; Endicott crude	MC252 crude	Kerosene, Diesel, Gas oil
Citations	Kang et al. 2014;Redman et al. 2017	Bera et al. 2018	Hammershøj et al. 2020; Trac et al. 2021