Title page

CRITICAL REVIEW OF IN VITRO DOSING METHODS FOR DIFFICULT-TO-TEST SUBSTANCES AND HYDROCARBON UVCBs

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Critical review of in vitro dosing methods for difficult-to-test substances and hydrocarbon UVCBs

Abstract

Alternative approaches to traditional animal testing are being promoted to support regulatory chemical risk assessments for environment and human health. The Organisation for Economic Development (OECD) has validated some in vitro test methods, but these methods are often suitable only for mono-constituent chemicals with a limited range of physicochemical properties. Most in vitro test methods are not suitable for poorly soluble, (semi)volatile, or multi-constituent chemical substances without significant methodological adaptations. In particular, substance of Unknown or Variable composition, Complex reaction products or Biological materials (UVCBs), including hydrocarbon UVCBs and petroleum substances (PS), can pose serious challenges for in vitro (eco)toxicity testing due to their complexity and variable chemical compositions. The choice of dosing method will depend on the purpose of the test as well as the physicochemical properties of the test substance. It remains difficult to establish and maintain stable exposures of PS in in vitro test systems due to different factors, including (1) the high surface area to volume ratios of multi-well plates that promotes sorption, (2) the open test wells that allow (semi)volatile constituents to escape or contaminate neighbouring plate wells, (3) the difficulty to analytically confirm exposure in small testing volumes and (4) the presence of lipids and proteins in biological media which bind PS constituents. This review maps the currently used dosing methods for hydrophobic and/or (semi)volatile chemicals and UVCBs in in vitro tests for environment and human health hazard assessments and outlines approaches and modifications to overcome various testing challenges associated with these test substances. Finally, research gaps are identified and recommendations made for future development of in vitro assays for UVCBs.

Keywords: difficult-to-test substances; exposure control; in vitro dosing; passive dosing; UVCBs

Introduction

Since the introduction of the 3Rs (Reduction, Refinement, and Replacement of animal testing) in the 1950s (Russell and Burch 1959), significant research has been carried out focusing on alternative approaches to traditional animal testing. These methods include any in vitro tests that can be used for screening, prioritisation, and/or to support grouping and read-across strategies for chemical risk assessment. Recently, there has been a surge of interest in how the 3Rs and in vitro methods could be better incorporated into regulatory frameworks. This is in part due to initiatives, such as the 'European

Union Roadmap for phasing out animal testing' and the 'US Food and Drug Administration's New Approach Methods Program', as well as recent legislation in the European Union, Canada, and the United States promoting the principles of the 3Rs (European Union 2010; Government of Canada 2025; United States Congress 2016). In the EU's Chemical Strategy for Sustainability, in vitro methods are prominently featured in discussions of the next generation chemical assessment (European Commission 2023a), and in vitro tests are part of the lower-tier screening assessment of potential endocrine-disrupting effects of chemicals, a hazard class that was recently integrated into the EU Classification and Labelling of Products (CLP) framework (European Commission 2023b).

The Organisation for Economic Development (OECD) has published validated in vitro test methods and defined approaches for their implementation (e.g., Guideline No. 497: Defined Approaches on Skin Sensitisation [OECD 2025]). Guideline validation, typically carried out through resource-intensive ring trials, is generally performed using discrete chemicals that have physicochemical properties suitable for maintaining stable exposure concentrations in biological test media. As such, standard in vitro test methods may not be suitable for poorly soluble, volatile, and/or multi-constituent chemical substances (i.e., "difficult to test chemicals") without significant adaptations to expand the applicability of these methods (Birch et al. 2019). In particular, substances of Unknown or Variable composition, Complex reaction products or Biological materials (UVCBs), including hydrocarbon UVCBs and petroleum substances (PS), pose serious challenges for in vitro (eco)toxicity testing due to their complexity, variable chemical compositions and physicochemical properties.

Petroleum Substances can contain hundreds to millions of hydrocarbon constituents, primarily aliphatic and aromatic hydrocarbons, but also heteroatoms of oxygen, nitrogen, or sulphur. The challenges in describing the composition of PS have spawned the field of petroleomics, demonstrating the difficulty of achieving complete characterization of these substances (Catalina et al. 2020). However, almost all PS constituents are hydrophobic, which makes them prone to sorptive losses in plastic multi-well plate assays. Key types of constituents, such as alkanes, cycloalkanes, and low molecular weight aromatics, are also volatile from aqueous solutions (Birch et al. 2018), which makes them prone to evaporative losses from open test systems (Birch et al. 2019; Escher et al. 2019; Kramer et al. 2010; Knöbel et al. 2012; Natsch et al. 2018; Peddinghaus et al. 2012). Riedl and Altenburger (2007) studied the relationship between physicochemical properties and unreliable exposure in microplate-based assays (24 well polystyrene plates). They found that chemicals with a log octanol-water partition coefficient (log K_{OW}) above 3 and log octanol-air partition coefficient (log K_{AW}) above −4 tend to exhibit unreliable exposure. Applying these findings to approximate the applicability domain of 24 well plate assays indicates that hydrocarbons are largely outside the range suitable for conventional plastic well plate systems, as illustrated in Figure 1. The data sets presented in Escher et al. (2019) and Fischer et al. (2018a) indicate that the same cut-offs also apply to 96-well plates.

Given the physicochemical properties of PS constituents, the challenges of testing them in vitro are mainly related to establishing and maintaining stable test concentrations and composition for the duration of the test. The method used to introduce a test substance to the exposure medium is herein referred to as the dosing method. The poor water solubility of PS, and the many constituents with variable properties, make the preparation of test solutions challenging and necessitates carefully designed dosing methods to establish initial test concentrations. Decreases in test concentrations during testing due to evaporative losses, sorptive losses, and well-to-well cross-over (i.e., inadvertent transfer of test material from one well to another [Birch et al. 2019]) may, if not controlled, compromise accuracy, reproducibility, and acceptability of the tests. Sorption to components in biological test media (i.e., lipids and proteins in whole cells or subcellular preparations and/or serum of cell culture media) or partitioning to the headspace of closed test vessels, may also reduce the bioavailability of test constituents and challenge the interpretation of results.

The OECD has published a guidance document with suggestions on how to amend standard aquatic ecotoxicity test methods to accommodate difficult-to-test substances such as hydrophobic chemicals, (semi)volatile chemicals, and UVCBs (OECD 2019). The overall specified strategy is to minimize sorptive and evaporative losses to achieve stable test concentrations, confirm the stable exposure at the end of the test, and, if necessary, control exposure by repeated dose, flow-through systems, or passive dosing (OECD 2019). Unfortunately, no such guidance document is available for the in vitro testing of difficult to test substances. This is urgently needed because the design of typical in vitro test systems, such as semi-open plastic multi-well plates, increases the potential challenges of maintaining a stable test concentration compared to standard aquatic ecotoxicity test systems. Efforts undertaken to maintain stable concentrations in larger-volume test systems in aquatic testing (e.g., repeated dose, flow-through systems, or passive dosing) are less common and more difficult for in vitro assays. Analytical confirmation of PS UVCBs can be challenging in in vitro test systems due to the small sample volume, the low solubilities of many specific constituents and the often complex in vitro testing media. Several challenges exist for the appropriate use of in vitro methods for hydrocarbon UVCBs (including petroleum) substances, and it is imperative to understand gaps in knowledge, adopting the most suitable methods, and developing novel testing strategies where necessary. This review maps the current use of hydrophobic, (semi)volatile chemicals and UVCBs in in vitro tests, outlining the approaches and test modifications used to overcome challenges associated with in vitro test systems for hydrocarbon UVCBs, and provides recommendations for future test strategies and research gaps. For the purpose of this paper, in vitro is defined as cells cultivated in an artificial environment outside of the organism. This included any mammalian or fish cell assay, recombinant microorganisms and aquatic embryotic tests in a non-protected life stage used as replacement of live animal testing.

Methodological Approach

Literature review methodology

This literature review had two purposes: 1. to obtain an overview of the state of the science on in vitro dosing methods for UVCBs; and 2. to evaluate the challenges and applicability of these methods. Two complementary search strategies were combined to ensure that sufficient relevant literature was included without introducing bias (Figure 2). A keyword search was performed to capture as many papers relevant to in vitro and dosing methods as possible. This search was further supplemented with a targeted citation search focused on the state of the science of dosing methods designed to establish and maintain defined exposure concentrations. A detailed description of the search strategies is given below.

Keyword search strategy

The purpose of this search was to identify any in vitro assay assessing a human or environmental toxicity endpoint where the test substance had one or more of the difficult-to-test properties (i.e., (semi)volatile, hydrophobic and muti-constituent). Specifically, we aimed to find descriptions of in vitro experiments that were intentionally designed to accommodate these difficult-to-test properties. In this context, we define “in vitro” as studies using isolated primary cells, cell lines, microorganisms used for specific mode of action (e.g., “mutagenicity” [Table 1]), and non-protected life stages of aquatic organisms (e.g., zebrafish embryos [Table 1]). The search string “UVCB AND in vitro” did not capture much of the literature as the term UVCB is seldom used in descriptions of test substances. Therefore, petroleum was used representing a UVCB of high interest. As one of the main challenges with UVCBs is that they contain multiple constituents, “multi-constituent” was used as an alternative search term, either in combination with different dosing methods or in combination with the properties “volatile” or “hydrophobic”. Although In vitro is an indexing term in PubMed and provides broad coverage for this topic, additional search terms were included to ensure relevance to (eco)toxicity testing. These included extra search terms for mutagenicity and embryos (Table 1) to cover bacterial and embryo assays, respectively. The tool AbstractSifter v7 (Baker et al. 2017) was used to compile the search terms based on key words related to assay categories, test compounds and dosing methods (see Table 1 for list of search terms used). The two search engines PubMed and Web of Science were used to retrieve literature with the same search terms on March 14, 2024. The resulting papers were imported and screened using the SWIFT Active Screener tool (Howard et al. 2020).

Firstly, two separate independent reviewers performed a Level 1 screen for each paper based on title and abstract to identify and include only papers describing in vitro assays used for testing substances

that were either UVCBs, hydrophobic or (semi)volatile. In vitro assays identified in this review included any assays using cells where a toxicological response was the endpoint, zebrafish embryos up to 120 hr post-fertilization, and bacterial assays for mutagenicity and basal toxicity. Antimicrobials, antifungals, and antiparasitic assays were considered outside of the scope, as well as plant cell lines for assessing herbicidal effects. Ex vivo (i.e., skin grafts or other organ tests) and in chemico tests (i.e., enzymatic assays) were excluded from the search. A few papers focused on invertebrate toxicity testing were included during the screening because the applied dosing methods mimicked in vitro testing scenarios (i.e., small volume aquatic test systems). The Level 1 screening was supplemented by the machine learning algorithm of SWIFT Active Screener software, and 54 highly relevant papers (identified by expert judgment to be relevant for in vitro testing of difficult-to-test substances) were used as positive seeds to train the algorithm. The algorithm ensured that the most likely relevant papers were screened first; thus, the screening was stopped after checking 1250 of the 4285 papers, as this corresponded to a 95% recall of relevant papers estimated by the algorithm.

Level 2 screening was conducted by checking the "materials and methods" chapter of each paper for the description of the (eco)toxicity assay, toxicological endpoints and respective assays used, the test system, the dosing method, and the test substance and this information was extracted to an Excel spreadsheet (see online supplementary material S1). Information on the vessels, exposure media and modifications performed, together with exposure verification and loss processes information, were also included (see online supplementary material S1). If either the assay, test system or dosing method was not described sufficiently for identification, the paper was excluded. Also, papers where the test substance was not recognized as either (semi)volatile, hydrophobic or UVCB were excluded. The inclusion and exclusion criteria used for Level 1 and 2 screening is summarized in Table S1 (see online supplementary material S2).

Information on the in vitro assays described in these papers were extracted to a database with one row per bioassay to give an overview of the in vitro assays used for UVCB substances in the literature. This information is published in the Zenodo open data repository (Wennberg et al., 2026).

Targeted citation search strategy

A targeted citation search was conducted to select articles that address and/or contribute to improving the exposure of petroleum UVCBs in in vitro tests with an emphasis on hydrophobic organic chemicals (HOCs), (semi)volatile organic chemicals (VOCs), and UVCBs containing HOCs and/or VOCs. The bibliography from the CEFIC ECO 36 project "Paving the way for QIVIVE – from nominal to free to cellular concentrations in in vitro assays"(Birch et al. 2019; Escher et al. 2020) was screened to select key papers to address these topics. From the selected 40 publications, six papers were selected based on (1) relevance: challenges in or improvement of in vitro tests is the focus of the paper; (2) sufficient

age for obtaining citations: >10 years except one newer study in which references were also considered; and (3) a certain coverage of active research groups. The rationale for this selection was that newer papers, addressing and improving the exposure control of in vitro assays for petroleum UVCBs, likely will have cited at least one of these six papers. The six papers were 3 key publications focusing on challenges related to losses (Heringa et al. 2004; Riedl and Altenburger 2007; Birch et al. 2019) and 3 key publications focusing on exposure control (Kramer et al. 2010; Smith et al. 2010a; Tanneberger et al. 2010). A citation search was performed on these papers in March 2024. These papers had in total 411 citations (not corrected for overlaps), and a screening was performed to select papers that address exposure of hydrophobic or (semi)volatile chemicals in in vitro tests or exposure control methods in aqueous tests in general. This screening led to 150 selected papers.

Sub-selection of experimental studies

A database of in vitro studies, captured in the two literature searches, was created based on the following selection of papers: From both literature searches, papers were selected that (1) describe an experiment with an in vitro test set-up including a toxicity endpoint, (2) conducted the test with a difficult to test substance, and (3) describe the dosing and test set-up in sufficient detail. This refinement excluded papers describing other toxicity tests using small volume aquatic organisms, bioaccumulation or biotransformation, IVIVE, partitioning studies, irrelevant test chemicals (i.e., neither (semi)volatile, hydrophobic nor UVCB), and papers discussing in vitro results from experiments described elsewhere. The details of the experiments described in these papers were compiled in a database, with one entry per study, and a study being a set of assays applying the same dosing method. Thus, one paper can include multiple studies, and one study can involve multiple in vitro assays and multiple test substances. Only test substances and bioassays of relevance to this review were included in the database.

Summary of the literature search outputs

An overview of the number of papers screened in the two literature searches are summarised in Table 2, and the included papers can be found in the SI. The two approaches had slightly different aims, with the keyword search focusing on the in vitro dosing methods, and the citation search focusing on the losses and exposure controls. Surprisingly, this resulted in only 19 papers overlapping between the two search strategies.

Of the included papers from the keyword search, 165 described cell-based in vitro test systems, 39 described various aquatic embryo tests, and 49 bacterial or yeast assays, nine described multiple test systems and two described tissues. Multiple assays were reported in many of the papers, and the

assays and endpoints are described in more details in a database accessible at Zenodo (Wennberg et al., 2026).

Most of the experimental papers from the targeted citation search described studies on mono-constituent chemicals (57 of 71 studies), while the keyword search had a wider range of test substances (51 % UVCB, 25% mono-constituent, 24% undefined mixtures from environmental samples or reaction products). Plant-derived UVCBs as a substance category was more frequent than PS UVCBs (26% plant-derived vs 17% PS UVCBs) when considering all studies. The substances were categorized based on difficult to test properties (DTTP), including volatility (log K_{AW} > −4), hydrophobicity (log K_{OW} > 3), or both, based on the cut-off criteria for 24 well plate assay applicability domain (Figure 1). For many of the plant derived UVCBs, there was no information on these properties, and they are listed as "UVCB dttp NR" in Figure 3. Solvent carrier was the most frequently used dosing method irrespective of DTTP, passive dosing was the second most frequently used for hydrophobic substances, gas phase exposure was the second most used for (semi)volatile substances and water or media accommodated fraction (WAF/MAF) was the second most common for substances that were both (semi)volatile and hydrophobic (Figure 3). Although most in vitro assays were performed in well plates (56%), other test vessels were also used, such as agar plates for the standard AMES test, well plate inserts for cells, plastic or glass dishes for larger exposure volumes, and glass vials for sealed exposures. The in vitro assays used in these studies are summarized in Figure S1 (see online supplementary material S2). They included bacteria or yeast systems for mutagenicity or endocrine testing, respectively ("bacteria or yeast"; Figure S1), cell lines or primary cells derived mainly from humans, other mammals or fish ("cell"; Figure S1), fertilized eggs from fish or other aquatic species, most commonly zebrafish ("embryo"; Figure S1), or excised tissue/tissue slices ("tissue"; Figure S1).

Dosing methods used in in vitro assays for substances with difficult-to-test properties

The different dosing methods aim at exposing the biological test system to either all components of the UVCB (i.e., via a solvent carrier, passive dosing, or particle carrier), the fraction of the UVCB that is soluble in a particular medium (i.e., via a WAF/MAF), the volatile fraction (i.e., gas phase exposure), or the fraction that partitions to organic solvents (i.e., solvent extraction). The dosing method implemented may depend on the research question and whether the investigation focuses on the whole substance or a particular fraction, constituent, or group of constituents. Depending on the nature and chemical complexity of the UVCB, a toxicity assessment relevant to a given exposure

scenario could require combinations of experiments to collect both whole substance and constituent information.

The principles of the most frequently reported dosing methods are illustrated in Figure 4 (with the number of studies for each method) and are discussed in more detail in the subsequent sections. Some methods were part of the original search strategy (Table 1) while other methods emerged during the review process (e.g., gas phase exposure, dosing with particle carriers). In addition to the methods in Figure 4, studies using direct addition/other dosing methods (13% in Figure 3) included dosing without solubilization either as an emulsion (4 studies) or a suspension (2 studies), direct addition of the test substance to test media from stock solutions prepared in media (i.e., distilled water or buffer solutions [9 studies] or without the use of stock solutions (8 studies), or short term exposure of cells to the test substance directly [1 study]). These studies are not further described herein but can be found in SI together with all the other in vitro studies with detailed dosing methods.

While dosing methods were typically selected to suit the properties of the substances, the limitations of the dosing method often did not appear to be a critical consideration, and only 44% of the studies included exposure verification by chemical analysis of the test medium.

Solvent carrier

Solvent carriers ("co-solvents") are widely used in both in vitro and in vivo studies to dose chemicals with low aqueous solubilities (i.e., hydrophobic chemicals). Dimethyl sulfoxide (DMSO) is the most common solvent due to its high solubilization capacity and biological assay compatibility. However, it is important to select an appropriate solvent based on the physicochemical properties of the test substance (i.e., polar or non-polar, multi-component), the biological system, and the dosing method (i.e., direct spike or pre-mixing in media; Green and Wheeler 2013; Luo et al. 2020; Tanneberger et al. 2010).

Solvent carriers enable precise nominal concentration control by a quantitative transfer of the UVCB mixture. Maximum concentrations and solvent types may be defined in standard test guidelines to avoid adverse effects caused by carrier solvents. For example, organic solvents (e.g., DMSO, methanol) are known to be non-acutely cytotoxic to at least 1% (v/v) in fish gill cell line assays (i.e., RTgill-W1) and the corresponding OECD TG 249 (OECD 2021) recommends solvent concentrations of ≤ 0.5% (v/v). However, the use of DMSO concentrations as low as 0.1% has been reported to interfere with vital metabolic pathways, demonstrating the potential impact a solvent may have on the outcome of an in vitro test (Nguyen et al. 2025). Justification for the choice of organic solvent and the final concentration in the exposure medium should be detailed. In certain assays, negative and solvent controls should be implemented to evaluate any solvent effects (e.g., as described in OECD TG 456 [OECD 2023]).

Solvent carrier was the most used dosing method in the experimental papers included here (Figure 3 and 4). The following solvents were most frequently used: DMSO (73% of studies), ethanol (9.6% of studies), methanol (7% of studies). Other solvents used alone or in combination included acetone, dichloromethane, acetonitrile, methyl cyclopentane, toluene, and petroleum ether. Dosing of these solvent carriers can be direct, where the solvent is added to the biological test system, or indirect, involving preparation of an intermediate solution with the solvent mixed in test medium before addition to the test system.

Although co-solvents are commonly used to dose organic chemicals, they can alter exposure by modulating compound-protein interactions in assay media, and thereby affecting bioavailability of test substances. For example, Luo et al. (2020) reported that applying greater dilution-factors of DMSO to extract PAHs from petroleum substances increased the extent of protein binding in cell culture media. Additionally, DMSO may exert physiological effects that can confound toxicological interpretations. It enhances intracellular uptake via membrane thinning and membrane-protein interactions (Gurtovenko and Anwar 2007; Notman et al. 2006; Williams and Barry 2012) and influences cellular processes such as G1-phase cell cycle arrest and estrogen receptor modulation (Takase et al. 1992).

Precipitation of poorly soluble constituents (i.e., test chemical with a limit of water solubility of <100 mg/L [OECD 2019]), can lead to heterogeneous exposure, increasing experimental variability (Hammershøj et al. 2020). While solvents like DMSO help to increase solubility in the stock/spike solution, a final concentration of solvents in the media of <1% does not increase the solubility in the exposure media or prevent precipitation. Moreover, heterogenous distribution when DMSO is spiked directly into cell culture wells may create concentration gradients that affect toxicity outcomes (Tanneberger et al. 2010).

Solvent extraction

Solvent extraction differs from solvent carrier in that only the fraction of the UVCB that is soluble in the organic solvent is added to the exposure medium, as opposed to the whole UVCB being added to the exposure media with the solvent carrier. The solvent carrier can be the same as the solvent used for extraction, however, more often the solvent used for extraction is evaporated and the sample extract dissolved in DMSO as solvent carrier for the in vitro assay dosing. In the reviewed literature, solvents used for extraction include cyclohexane, dichloromethane (DCM), DMSO, acetone, methanol, pentane, and hexane, either alone, in combination or in consecutive extractions. The most prominent solvents were DCM alone or in combination with other solvents (used in 38% of the studies) and DMSO alone or in combination (used in 25% of the studies). Solvents are selected based on the fraction of interest and the material it is extracted from. For instance, extraction of organic material from samples of particles from air is most often done using DCM alone or in combination with other solvents (as

summarized by May WE et al. (1992). These authors also found that mutagenicity of air particles and diesel particle extracts did not appear to be dependent on the solvent or extraction method used (May et al. 1992). Another example is the study of effects from polycyclic aromatic hydrocarbons (PAH) in petroleum substances where several methods were based on a combination or sequence of extraction in DMSO and cyclohexane (as described in (Cordova et al. 2023a; Gray et al. 2013; Grimm FA et al. 2016). Solvent extraction is used because PAHs preferentially extracted by DMSO are considered to be the 'bioactive' part of the petroleum substances. However, extracted samples might give different results than the whole substance as interactions between components in the UVCB can affect bioavailability. A study that compared defined mixtures of analytical standards with extracted PAH from four PS UVCBs found that extraction procedures, protein binding in cell culture media and dilution factors prior to in vitro testing can all contribute to determining the final bioavailable concentrations in vitro (Luo et al. 2020).

Water or Media Accommodated Fractions

For PS UVCBs, a commonly used method for preparing an aqueous solution for testing is a Water or Media Accommodated Fraction (WAF or MAF), where the UVCB is mixed with water or media at a defined energy depending on study design to allow the water-soluble constituents to partition into the aqueous phase. The aqueous phase is subsequently removed after a set mixing time, avoiding removing the insoluble fraction to reduce the likelihood of non-chemical or physical effects. If the WAF needs to be filtered to remove any undissolved emulsified components, then the resulting solution is a water-soluble fraction (WSF; Wheeler et al. 2020). Media Accommodated Fractions (MAFs) are prepared similarly to WAFs but with the respective test media being used for preparing the exposure solutions rather than using dilution water.

According to the Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR 2021) and OECD GD 23, the duration that WAFs need to be mixed and settled should be based on a preliminary study because WAFs can vary based on the nature of the UVCB substance and the method of preparation used. Water accommodated fractions are usually prepared in aspirator flasks using low-energy (LEWAF) mixing by stirring with sufficient energy to form a small vortex in the flask (typically using a magnetic stir bar) or by shaking for a fixed period (usually 24 hr). A high energy WAF (HEWAF) may be prepared using a high energy stirring method (e.g., using a hand blender), usually for a shorter period (e.g.,1 min). However, it has been reported that HEWAFs can be less toxic than LEWAFs due to oil droplets being present in the aqueous phase (Alloy et al. 2022). Consequently, LEWAFs are often the preferred method in regulatory testing scenarios as their lower-energy preparation minimizes droplet formation and is considered as more appropriate for assessing the innate chemical toxicity of the substance. Bilbao et al. (2022) suggested that 48 hr of stirring raw oils

to create WAFs was sufficient to achieve steady state concentrations of PAHs (primarily naphthalene related compounds). In addition, the authors concluded that the solubility of the PAHs increased with increasing temperature and that using a dispersant can also affect the solubility. Use of dispersants during WAF preparation (Chemically Enhanced WAFs, CEWAF) to facilitate the solubilization may introduce confounding factors, and any test employing a dispersant needs to include a dispersant-only control (Bilbao et al. 2022). DeMiguel-Jimenez et al. (2023) also argued that the effect of temperature on generating a saturated WAF is further exacerbated by the pour point of the test substance (i.e., viscous substances with higher pour point oils such as wax-rich oils compared to oils with lower pour points such as naphthenic acids; DeMiguel-Jiménez et al. 2023).

The advantage of WAF/MAF is the avoidance of the precipitation of less soluble constituents and the avoidance of the use of co-solvents. When preparing WAFs, partitioning and dissolution of constituents determine the ultimate mixture composition in the water, where the concentrations of water-soluble constituents are enhanced compared to those of less water-soluble constituents (Hammershøj et al. 2020). Water and media accommodated fractions may be more environmentally relevant though not necessarily the worst-case scenario for testing the PS UVCB, as it could mimic the partitioning dynamics seen in the environment. The ratio between the PS loading and water volume determines whether the least hydrophobic constituents are depleted in the PS. Therefore, when WAFs are prepared, the partitioning of compounds into the water phase does not change linearly with the loading concentration. Consequently, WAFs should not be prepared by serial dilution (Bluhm et al. 2016).

The technical guidance document on difficult-to-test substances (OECD 2019) recommends the use of WAFs and loading rates to estimate effect levels of UVCBs; this concept is also relevant for MAFs. As documented in Wheeler et al. (2020) and according to OECD GD 23, the process for toxicity testing of UVCBs should include: 1) Analytical characterization of the chemical composition of the UVCB; 2) Preparation of a separate WAF at individual loading rates; and 3) Reported toxicity thresholds from the loading rates (i.e., mass-to-volume ratio of the whole UVCB to test media). Passive samplers, including biomimetic extraction-solid phase microextraction (BE-SPME), can complement WAF studies, as they quantify freely dissolved concentrations of the UVCB in WAFs. For example, BE-SPME has been applied in the assessment of sublethal toxicity of petroleum substances in zebrafish embryo assays (Hedgpeth et al. 2019).

The OECD guidance document (GD 23) was recently adapted for the preparation of media-accommodated fractions (MAF) at microscale volumes to expose a defined mixture of aromatic hydrocarbons to mammalian cells, suggesting some utility of these approaches to accommodate difficult-to-test substances (Cordova et al. 2023b). However, WAF and MAFs may not be appropriate to maintain a stable concentration for all UVCBs tested in vitro due to losses via volatilization or from absorption to various compartments of the well. In addition, although small test systems are desirable

for efficient, high-throughput testing (i.e., 96- or 384-well plates), the small volumes utilized to expose cells in vitro may further exacerbate this issue.

The major difference between WAF and MAF is the complexity of the culture media that might affect partitioning and dissolution of test constituents. Most of the studies using MAF/WAF in this review conducted embryo tests (18 of 32). Only three of the studies covered in our literature search prepared a MAF using cell culture media, while the rest of the studies, using cells, prepared WAFs in ultrapure water and either added medium supplements afterwards or used high dilution factors in cell media. Cordova et al. (2023b) prepared MAF using cell culture medium with serum to expose mammalian cells to a defined mixture of 25 PAHs. As per the findings from F. Fischer et al. (2019), Cordova et al. (2023b) utilized the serum to *"compensate for losses in freely available concentration of reversibly bound* hydrophobic *chemicals"* in the exposure solution, with the objective to stabilize compound concentrations by "serum-mediated passive dosing".

Importantly, the dosing of in vitro tests using WAFs do not involve a means to stabilize concentrations during the test. Therefore, glass culture vessels were often used to prevent absorption to plastic. Studies using MAFs specified that the test vessels were sealed to prevent evaporation. If WAFs or MAFs are used, the need to replace the solutions during the assay should always be evaluated to ensure constant exposure and whether the effect of serum-mediated passive dosing would stabilize exposure in MAFs.

Passive dosing

Passive dosing uses a partitioning donor to establish and maintain constant concentrations of a test substance in aquatic and in vitro tests (Smith et al. 2010a; 2010b). A biologically compatible polymer is first loaded with the test substance and then added to the test system. High partition ratios between donor and test medium and carefully designed volumes of donor and medium ensure that freely dissolved concentrations are controlled and maintained in the test by equilibrium partitioning in cases where there are losses from test media i.e. due to volatilization or sorption. A series of multiple donor concentrations yield an equivalent series of freely dissolved test concentrations, which facilitates well-defined concentration-response testing (Vergauwen et al. 2015). When dosing mixtures, each constituent will partition between the donor and test medium according to its own partition coefficient, meaning that the proportion of each constituent in the test media will differ from its proportion in the loaded mixture (Hammershøj et al. 2020).

Silicones, particularly polydimethylsiloxane (PDMS), are the preferred polymers for passive dosing because they are available in various food- and medical-grade physical formats. Additionally, PDMS maintains its excellent partitioning properties also when in contact with various biological materials (Jahnke and Mayer 2010). Other suitable polymers have also been tested, such as ethylene vinyl

acetate (Lee et al. 2012). Various physical forms of donors have been used for passive dosing in in vitro tests. Silicone O-rings are commonly used because they easily fit into 24 well-plates and can be used without a direct contact between the passive dosing donor and, for instance, the cells or embryos (Bougeard et al. 2011; Maner et al. 2019; Massei et al. 2021; Oostingh et al. 2015; Smith et al. 2010a; Smith et al. 2013; Vergauwen et al. 2015;). Other formats include silicone disks (Roh et al. 2014), thin films at the bottom of wells with cells grown on inserts not touching the PDMS films (Booij et al. 2011; Kramer et al. 2010), and well-plate inserts used as hanging silicone disk holders (Gilbert et al. 2015). All of the in vitro studies covered in our literature search used silicone, with 68% in the format of O-rings, and the rest as PDMS discs or sheets.

Passive dosing is generally applicable to hydrophobic chemicals with log K_{OW} > 3; however, there is limited documentation regarding the application of passive dosing for chemicals above log K_{OW} 6 to 7. The constraints lie mainly in the loading of the silicone, as the silicone may not be able to absorb larger molecules. Two loading principles can be used to add UVCBs to the silicone. The first is the direct immersion of the silicone in the UVCB for maximum absorption of UVCB constituents. Lower loadings can be obtained by allowing shorter uptake times before the silicone is retracted and its surface wiped of excess test substance (Hammershøj et al. 2020). The second is to add a fixed amount of UVCB to the silicone for maximum absorption. It should be noted that if small amounts of UVCB are added to the silicone, it can be time-consuming to produce an even distribution in the silicone.

Gas phase exposure

Several test set-ups are described for gas phase exposure to volatile and semi-volatile compounds, aerosols, gases, and complex mixtures in in vitro test systems. These include tight flasks, gas sampling bags, closed chambers with saturated filter papers, gas-liquid equilibrium systems, and exposure chambers with steady state or controlled air flow exposures. The test system is exposed either in the culture medium, through gas-medium equilibrium, or at the air-medium interface (i.e., air-agar interface (Riedel et al. 2018), transwell inserts (Binder et al. 2022; Lestari et al. 2012; Li et al. 2024; Lin et al. 2014; Verstraelen et al. 2020;) or hanging drop (Liu et al. 2014) for cell cultures). Exposure at the air-liquid interface refers to cells grown on microporous membranes in transwell inserts with the basal surface in contact with culture medium and the apical surface exposed to air. Compared to traditional compound addition to the medium and exposure under submerged conditions, exposure at the air-liquid interface has the advantage of higher physiological relevance for inhalation studies. In addition, it provides more efficient delivery of airborne contaminants, avoiding losses due to evaporation, degradation (from hydrolysis), and substance reactions with the constituents of the culture medium (Liu et al. 2014). Depending on the exposure system, the cells can be exposed to defined flow rates of aerosol, the gas and particulate phases can be separated (Binder et al. 2022), and cell deposition can

be monitored and quantified (Binder et al. 2022; Lestari et al. 2012; Li et al. 2024; Lin et al. 2014; Verstraelen et al. 2020).

The exposure system flow direction seems to be of importance for efficient substance deposition. For instance, perpendicular flow systems were not appropriate for gasoline exposure to cells due to low deposition of gasoline to cells and media (Verstraelen et al. 2020). The low deposition was attributed to aromatic or saturated hydrocarbon structure, high hydrophobicity, and low concentration for measurements. Therefore, the authors also performed additional experiments for comparison with passive exposure of the cells under the air-liquid interface by placing various gasoline volumes in open glass vials or petri dishes inside a humidified desiccator in a climate chamber. This showed that a higher compound deposition on cells and media was achieved with a dose-dependent decrease in cell viability (Verstraelen et al. 2020).

A gas phase in vitro exposure system with a diffusion vial has been used for exposure of A549 alveolar cells to volatile organic compounds acrolein and methacrolein, water-soluble secondary gases produced by photochemical reactions (Lin et al. 2014). Although exposure to acrolein led to dose-dependent effects, it was highlighted that the system was not appropriate for chemicals with very high and low vapor pressure, hygroscopicity, and polymerizability (Lin et al. 2014).

Particle carries, including nano carriers

Carriers, including nanoparticles, have been used for the encapsulation of hydrophobic, (semi)volatile, and easily degradable compounds to improve stability, cell permeation, and controlled compound release and delivery to target cells in aqueous environments. Lipid vesicle incorporation has previously been used to increase the delivery efficiency of a model petroleum sample (i.e., a matrix-reconstituted sample of distillate fractions from crude oil spiked with radiolabeled tracer substances) to cells for genotoxicity studies and compared to emulsification and solvent delivery (von Hofe et al. 1986). Higher dose-dependent delivery of the substance was observed for liposomes compared to emulsion or acetone delivery leading to increased cytotoxicity. Polymeric nanoparticles such as chitosan (Onyebuchi and Kavaz 2019; Rajivgandhi et al. 2020), N, N, N-trimethyl chitosan (Onyebuchi and Kavaz 2019) and Eudragit L100, nanovesicles (glycerosomes; Vanti et al. 2020), and solid lipid nanoparticles (Rodenak-Kladniew et al. 2023) have been used for the encapsulation of essential oils to preserve the main components from degradation and improve release and delivery. The studies often showed increased stability, controlled release, and enhanced cytotoxicity or antiproliferative activity of particle-loaded essential oils compared to free oil.

The focus of such studies is the potential applicability for cancer treatment; the release kinetics have been performed in buffered systems (e.g., phosphate buffered saline) at low pH to mimic the behavior after release in the cells (acidic environment in tumor cells or endolysosomes). However, the

particle/carrier behavior, stability, and compound release kinetics can be affected by the exposure media composition or by the presence of serum or other proteins, which are often not considered. There are also uncertainties about the optimal size, type of carrier, and substance properties for optimal loading, release, and delivery. Therefore, although the use of carriers as a delivery methodology shows promise, further development and validation studies are needed to address the suitability for UVCBs of different properties.

Dosing methods advantages and disadvantages

The different dosing methods are based on different principles and result in different UVCB constituent test compositions. While the solvent carrier aims to quantitatively transfer the UVCB to the test system, partitioning processes may change the composition and relative proportions of individual constituents in the test. Solvent extraction, WAF/MAF, and passive dosing modify the UVCB composition in different ways depending on their partitioning into solvent, water, test media, and silicone. However, passive dosing is the only method that controls constituent concentrations and mixture composition in the test because of the high capacity of the dosing phase to buffer the test substances when used in 24-well plates (see discussion in the next sections). Table 3 lists the applicability, advantages, and disadvantages of the different dosing methods.

Maintaining and measuring substance concentrations during the exposure phase

Toxicity tests are generally conducted to establish the relationship between dose/concentration and observed effects. A clearly defined and stable exposure during testing is therefore crucial (OECD 2019). In most in vitro studies reviewed here (56% of studies), the reported toxicity is related to the nominal test concentration (i.e., the amount of test substance added to a fixed volume) which is of limited use as the actual exposure concentrations are not reported. Specifically, in typical semi-open multi-well plate systems, constituent concentrations will decrease due to evaporation, sorption and/or degradation (Birch et al. 2019). According to the OECD guidelines for aquatic toxicity testing, a decline in concentration of ≥ 20% for mono-constituent substances would warrant a need for measures to prevent losses (OECD 2019). The same threshold is reasonable to follow also for in vitro testing. Furthermore, some in vitro tests are conducted with exposure media rich in proteins (e.g., amended with serum). Hydrophobic compounds partition to these dissolved organic carbons, which will lead to a lower freely dissolved concentration even if total aqueous concentrations are not affected. If it is feasible to measure the internal cell or organism exposure, then that is preferred as the most relevant dose metric in in vitro tests (Groothuis et al. 2015). Otherwise, freely dissolved concentrations should

be used as the dose metric in all cases where >20% of the test chemicals are bound to serum (Groothuis et al. 2015). Improved exposure control for petroleum UVCBs requires (1) that exposure concentrations are monitored throughout the test duration, (2) that the test system is designed to minimize losses and/or (3) that some mechanism or action is taken to stabilize concentrations against losses (pre-equilibrium of test vessel, flow-through systems or passive dosing).

Avoiding losses due to volatilization

Volatile losses from aqueous test media are not controlled by the vapor pressure of chemicals, but rather by their air-water (or air-medium) partition ratios. Since a higher serum content in the medium can be used to reduce volatile losses (Fischer et al. 2018a; Kramer et al. 2010; Smith et al. 2010a;), it has been suggested to use a high serum content to ensure stable exposure concentrations in in vitro tests – the so-called 'serum-mediated passive dosing' (Fischer et al. 2018b; F. Fischer et al. 2019; Gilbert 2015). In Figure 5, the applicability domain where a higher serum content can stabilize concentrations in well plates is shown in light yellow (Birch et al. 2019). It is clear, that while a higher serum content can keep some PAHs in the medium, it is not applicable for alkanes and cycloalkanes which have log K_{AW} values exceeding −4.4. (F. Fischer et al. 2019). A necessary step to avoid losses of hydrocarbon constituents in PS UVCBs is to use closed test systems.

In the literature, well plates have been sealed using aluminum foil and a glass plate pressed on top (Schreiber et al. 2008), aluminum foil with a Viton sheet pressed on top (Kramer et al. 2012), or a PTFE/silicone rubber sealing mat (Stalter et al. 2013). However, an algal growth inhibition test showed that for kerosene (boiling point range 125–287°C), even small air bubbles in a closed system (0.5 ml volume) reduced water concentrations (water concentration of 90% reduced to 71%; Mayer et al. 2000). To avoid this, Stalter et al. (2013) used a headspace-free well plate setup for volatile disinfection by-products. Closing well plates also prevents the cross-over of chemicals to adjacent wells. Cross-over is a particular challenge for semi-volatile chemicals, which can lead to effects in control wells (Beresford et al. 2000; Thellen et al. 1989) and measurable concentrations in neighbouring wells (Birch et al. 2019; Lee et al. 2022; Schug et al. 2020). Figure 5 illustrates (purple striped and hatched area) the chemical space where cross-over has been observed (Birch et al. 2019; Lee et al. 2022). While sealing plates with aluminium well plate sealing tape eliminated cross-over in the study by Birch et al. (2019), covering the well plate with aluminium foil alone was insufficient to avoid cross-over in the study by Schug et al. (2020).

The literature review showed that volatile losses are generally not taken into account in studies, which can lead to invalid results. Only 29 of 172 in vitro studies with (semi)volatile chemicals reported the use of closed test vessels, and only one reported using a headspace free set-up. It is, however, clear that closed test systems with no- or limited headspace is crucial for testing PS UVCBs containing

(semi)volatile constituents such as linear and branched alkanes and cycloalkanes, but also for a range of aromatic hydrocarbons (mono-, di-, tri-aromatic and cycloalkane aromatic hydrocarbons).

Measures to avoid losses due to adsorption

Most petroleum hydrocarbons are hydrophobic, and even in closed test systems, losses can be substantial due to sorption to plastic well plates, inserts, and adhesive foil glue (Chlebowski et al. 2016; Kramer et al. 2015; Schreiber et al. 2008; Stadnicka-Michalak et al. 2021). The partitioning process is reversible so that if equilibrium shifts, through uptake and metabolism in cells, the substances can partition back into the medium (Madureira et al. 2014). The presence of other sorptive phases, such as lipids and serum in the medium, can reduce sorption to plastic (Kramer et al. 2012; Madureira et al. 2014; Schirmer et al. 1997; Zhang et al. 2020), and whether chemicals are sorbed to the medium or plastic does not necessarily affect the uptake in cells (Hestermann et al. 2000; Schirmer et al. 1997).

Reduction of sorptive losses can be obtained by avoiding plastic materials in tests. Despite being more costly than plastics, glass is the most used alternative material for in vitro testing of hydrophobic chemicals (Knöbel et al. 2012; McDermott et al. 2007; Schreiber et al. 2009). Custom-made Teflon well plates (Kwon et al. 2007), and a stainless-steel exposure chamber (TransFEr) have also been used (Schug et al. 2018; Schug et al. 2019). Plexiglass® (polymethyl methacrylate) has been investigated as a material type that reduces sorption (Schreiber et al. 2008), but to our knowledge, it has not been used in in vitro tests. While glass well-plates or vials are not yet commonly used in in vitro testing, they are crucial test amendments to minimize sorptive losses of hydrophobic test chemicals and mixtures.

Exposure control

Because reliable toxicity testing depends on maintaining stable exposure concentrations, it is important to consider how test design and dosing strategies can minimize losses while maintaining chemical exposure. While modifying test designs can help reduce chemical losses, such measures may not be sufficient to ensure stable test concentrations for very volatile and hydrophobic chemicals. In these cases, the test chemicals may have to be replenished during the experiment. However, repeated dosing in tests can introduce more variability in exposure conditions when losses occur rapidly (Broeders et al. 2015). To address this challenge, passive dosing systems have been developed to continuously stabilize exposure concentrations by replenishing concentrations during losses (Booij et al. 2011; Kramer et al. 2010; Smith et al. 2010a; Smith and Schäfer 2017; Vergauwen et al. 2015).

For hydrophobic chemicals (log K_{OW} > 3) passive dosing has been shown to improve exposure control. Employing passive dosing in in vitro bioassays with PAHs resulted in a log-linear (R^2>0.8), log K_{OW}-dependent increase in response sensitivity compared to conventional dosing (Bougeard et al. 2011). Achieving stable concentrations requires the polymer phase to have a sufficient capacity to prevent

depletion during test incubation. Otherwise, it can be necessary to replace the dosed polymer periodically (Niehus et al. 2018; Vergauwen et al. 2015). Using plastic test vessels can cause the test chemicals to partition into the vessel material, which can also cause dynamic concentration profiles. Therefore, to achieve the most stable exposure conditions it is therefore recommended to reduce losses by using closed glass well plates or vials and to pre-equilibrate the vessel and test medium with the substance (from the polymer) prior to test initiation.

Exposure confirmation

Exposure confirmation is necessary for the correct interpretation of toxicity data. Freely dissolved concentrations of hydrophobic chemicals in tests have been determined by e.g., solid phase micro-extraction (SPME) techniques (Broeders et al. 2011; Henneberger et al. 2019; Heringa et al. 2004). In one study, SPME fibres were pierced through a Viton sheet, exposed to the solution in each well of the well plate, and then pulled out of the Viton and analyzed (Heringa et al. 2004). For non-volatile chemicals it is possible to transfer the medium to separate vials for extraction with SPME (Henneberger et al. 2019). If freely dissolved concentrations cannot be obtained (e.g., highly hydrophobic chemicals), the measurement of total concentrations may be more accessible, e.g., by liquid-liquid extraction (Cordova et a. 2023b). Total concentrations can then indicate stability over time.

Exposure models have been developed to predict freely dissolved concentrations in tests. While partitioning models can describe the distribution of hydrophobic and (semi)volatile chemicals between phases of a test system, using them for volatile losses from in vitro tests leads to higher uncertainties because the headspace is not well defined in an open system (Armitage et al. 2014; Proença et al. 2021; Stadnicka-Michalak et al. 2014; Stadnicka-Michalak et al. 2021). In one study, measured losses corresponded to a headspace of 50 to 100 times the nominal headspace of the well (Armitage et al. 2021). While exposure models can be a good supplement to analytical exposure assessment, they are currently only suited for closed systems. Models may also be of limited used in the case of UVCBs for which composition remains ill-defined. However, exposure models could provide insight into the actual exposure of test systems in the case of UVCBs whose composition and partitioning behaviour are better known, via the modeling of specific fractions or representative constituents of the whole UVCB (Deglin et al. 2026).

In vitro - in vivo extrapolation (IVIVE)

In vitro-in vivo extrapolation (IVIVE) is crucial in leveraging in vitro data to estimate in vivo outcomes. Such extrapolations frequently utilize physiologically-based pharmacokinetic (PBPK) modelling to either extrapolate an in vitro dose to an external exposure concentration or to estimate in vivo whole-organ absorption, distribution, metabolism, and excretion (ADME) properties (Chang et al. 2022). Such

PBPK models are based on measured (i.e., experimentally generated values) or calculated (i.e., quantitative structure-activity relationship [QSAR] modelled values) parameters related to ADME.

Even for single chemicals, generating in vitro test data to parameterize PBPK model parameters requires careful exposure control for substances with difficult-to-test properties. For example, researchers have applied passive-dosing methods to improve the generation of biotransformation data for highly hydrophobic chemicals. These methods minimize co-solvent effects and ensure the complete solubilization of the test material; however preventative steps to reduce losses should still be undertaken (Kwon et al. 2009; Lee et al. 2012; Lee et al. 2014). The ability to apply passive dosing in in vitro investigations also allows for determination of the free test concentration, which when combined with internal or target dose information can improve IVIVE model estimates (Escher and Hermens 2004; Groothuis et al. 2015; Gülden and Seibert 2005; Kwon et al. 2007; Kramer et al. 2009) by explicitly accounting for non-specific binding (Altenburger et al. 2018; Broeders et al. 2013; Dupraz et al. 2019; Fischer et al. 2016; Altenburger et al. 2018; Dupraz et al. 2019; M. Fischer et al. 2019; Heringa et al. 2004; Smith et al. 2013;).

Substance of Unknown or Variable composition, Complex reaction products or Biological materials and other difficult to test substances face unique challenges regarding IVIVE, as both QSARs and in vitro estimation of parameters have historically been developed/utilized for single constituent chemicals. For example, in silico methods rely on the chemical structure of the molecule of interest, which does not apply to UVCBs. Even in instances where all constituents of a UVCB or mixture are identified and QSAR values can be generated for the individual constituents, there is ongoing discussion on the best way to model the combined effect of the mixtures (Belfield et al. 2023). In the case of experimentally derived parameters, it is typically assumed that test materials in an in vitro system behave the same way as in vivo. However, the chemical profile of UVCBs can change in in vitro systems due to the potential loss of (semi)volatile constituents and/or adsorption of lipophilic fractions to test vessels and components of in vitro media such as serum. Additionally, these differences can be dependent on the concentration of the test material (Luo et al. 2020). For UVCBs, research on the characterization of these dose metrics and the identification of appropriate extrapolation factors remains limited, and the potential influence of chemical-to-chemical interactions on the bioavailability of individual constituents is still not well established (Luo et al. 2020). Furthermore, approaches for addressing such interactions, as well as the applicability of grouping strategies in IVIVE for estimating target in vivo doses or potential mixture effects, remain to be determined.

Considerations for study design and choice of an in vitro dosing method appropriate for the UVCB

The key considerations for selecting appropriate in vitro assays and dosing methods for UVCBs are summarized in Figure 6, with the intention that the study design should be fit for purpose and not be more complicated than required. Thus, the first question is "what is the purpose of testing?". The route of exposure (e.g., human or environmental concern, inhalation, ingestion or direct contact) will set some limits to the type of test system (e.g., fish embryo, cell lines) and the dosing method (e.g., simulate environmental partitioning, volatile fraction or whole substance).

Some of the endpoints also require specific test systems (e.g., such as mutagenicity using AMES test, acute or developmental toxicity using the FET test, or use of cell-based assays requiring rich medium). Furthermore, if the testing is for regulatory purposes, there may be restrictions on choice of endpoints and permitted modifications of test set-up particularly when adhering to Good Laboratory Practice (GLP). However, the standard regulatory test guidelines most often do not consider difficult to test properties such as those of PS UVCBs, thus modifications of standard tests are necessary to document an endpoint for regulatory purposes. If the intention is to investigate, for example, specific modes of action, compare responses between substances, or screen for new endpoints, the test set-up can be either pre-defined or very flexible to accommodate the physicochemical properties of the test substance.

Table 3, which includes information on the applicability, advantages and disadvantages of the different dosing methods, and Figure 7, that shows a simplified representation of the applicability domain of the dosing methods, can aid in the selection of the right dosing method and test set-up for a given test. The dosing method should be selected based on the combined physicochemical properties of all the constituents of the UVCB substance. The boxes in Figure 7 represent the operational chemical space of the dosing method, however, as the reviewed literature did not specify exact cut off criteria for volatility and hydrophobicity, the chemical space is indicated in a qualitative way, relative to each other. The blurred edges of the boxes indicate that chemical interaction between constituents or modifications of the dosing methods can affect the applicability domain. Direct addition, solvent carrier and passive dosing are used for transferring the whole UVCB to the test medium, and the choice between these methods is related to the hydrophobicity of the substance. For WAF/MAF, gas phase exposure and solvent extraction, the boxes represent the constituents that will be transferred to the test medium with this method. Without modification, (semi)volatile constituents might be lost before partitioning to the test medium using WAF/MAF and solvent extraction, however, modifications of

WAF/MAF systems can be done to accommodate more volatile substances, (i.e. Parkerton et al. 2023; Redman and Parkerton 2015). The choice of solvents, for solvent extraction, determines which constituents are extracted, so the green box in this figure could be in different positions or a different size.

In addition to the dosing method considerations, maintaining exposure concentrations also relies heavily on the design of the test system. As previously discussed, hydrocarbon constituents are typically hydrophobic, (semi)volatile, or both, making them poorly suited for testing in unsealed, plastic multi-well plates. Ideally, testing should be conducted in closed test systems made of glass with limited headspace. A decision tree that supports the design of appropriate dosing strategies for in vitro studies, is provided by the "Better In Vitro Dosing Consortium" (www.betterinvitrodosing.com/).

Another consideration is that sufficient volumes of test solutions are needed for analytical confirmation of exposure, or if extensive analytical confirmation is not possible, that the test system is designed in a manner to allow for robust exposure modelling.

Ultimately, analytical exposure confirmation is the preferred approach to verify that the substance composition and constituent concentrations are stable within the test system. For a UVCB where a comprehensive targeted analysis is not feasible, comparing chromatograms (e.g., using non-targeted analysis) between initial and definitive samples can demonstrate exposure stability, or reveal constituent specific losses. If this verification confirms that losses are minimal and the test system maintains stable concentrations and composition, the test can be conducted, and the results may be expressed in terms of nominal concentrations or loadings. However, if losses are considerable, the dosing method and test system should be reevaluated and improved where possible. If further modifications are not possible, a combination of analytical measurements and modelling can be used to determine time-resolved test concentrations, for example by measuring free concentrations with SPME and partitioning or in vitro mass balance models.

Research gaps and concluding remarks

This review demonstrates that in vitro testing of difficult-to-test substances is feasible when existing dosing methods are appropriately aligned with the properties of the substance and the test system design. However, UVCBs and multi-constituent substances introduce additional complexity because their constituents can occupy a broader chemical space than single discrete chemicals, leading to wider physicochemical behaviour. This challenge is compounded by the fact that UVCBs may be only partially characterized. Therefore, when the properties of the uncharacterized fraction are unknown or fall outside the applicability domain of a given method, selecting an appropriate dosing approach becomes difficult. Such compositional uncertainties directly affect confidence in the resulting test outcomes (Deglin et al. 2026).

Substance composition and its associated uncertainty and variability should be explicitly considered when choosing dosing methods for in vitro testing. Clear reporting of assumptions, uncertainties, and any methodological adaptations will be essential for improving reproducibility and comparability across studies. While applicability domains developed for single discrete chemicals can provide an initial framework for UVCB dosing decisions, interactions among UVCB constituents may shift these boundaries and warrant further investigation. Transparent justification of dosing method selection, test system configuration, and any deviations from standard protocols is therefore recommended.

Although Figure 7 illustrates the relative domains of different dosing methods, precise cut-off criteria defining practical limits for water solubility, hydrophobicity, volatility, and molecular size remain poorly defined. Each dosing approach can also be adapted to better accommodate different chemical characteristics which may shift the relative boundaries presented in Figure 7. For example, this can be achieved through solvent selection (i.e., polar or non-polar) for solvent carrier- or extract-based dosing, by adjusting serum content in media for MAFs, or by modifying donor polymer characteristics and loading for passive dosing. At the same time, it is important to recognize that substances with extreme properties (e.g., very low solubility, very high or superhydrophobicity) may not be bioavailable in vivo and may reasonably fall outside the scope of in vitro testing.

There are other factors related to UVCB dosing methods that may warrant further investigation. For instance, when preparing MAFs in cell culture media, it is unclear whether separate MAFs must be prepared at each loading level (as is recommended for WAFs) to maintain a stable and representative composition. Serial dilution from a single MAF could potentially change the relative proportions of constituents, but this has not yet been characterized for preparations in culture media. Another example is the application of in vitro systems to simulate metabolism (e.g., with S9 fractions, microsomes, or recombinant enzyme systems) using passive dosing setups, where the parent compound will be maintained at controlled free concentration, while the concentrations of metabolites may progressively increase. It should also be explored if a suite of multiple dosing approaches would provide more representative results for complex UVCBs rather than optimizing a single method. Importantly, dosing methods must be considered in conjunction with the overall test setup and strategies to minimize losses by adsorption, volatilization, or crossover are essential for improving data quality.

Finally, allowing flexibility for well-justified non-standard or adapted methods can help ensure that in vitro testing more comprehensively represents the chemical range of the UVCB (Deglin et al. 2026). Many OECD methods were not designed for substances exhibiting wide variation in volatility, hydrophobicity, or constituent diversity, and applying them without modification may yield unreliable results. The development of clear frameworks outlining when non-standard setups (e.g., sealed systems, passive dosing, modified media, multi-approach dosing) are scientifically justified would

support more fit-for-purpose testing of UVCBs. Ultimately, harmonizing reporting expectations, improving transparency in method selection, and integrating refined dosing and exposure-verification procedures into regulatory guidance will enable more consistent, exposure-relevant, and regulatory-ready in vitro toxicity data for complex substances, supporting both human health and environmental risk assessments.

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Figure 1. Chemical space plot positioning petroleum hydrocarbons (n=53) relative to the applicability domain of 24 well plate assays (Riedl and Altenburger 2007). The representative petroleum hydrocarbons (green and blue datapoints) are plotted according to their hydrophobicity (log K_{OW}) and volatility (log K_{AW}; Birch et al. 2018). All hydrocarbons are outside the applicability domain of a 24 well polystyrene plate algal bioassay. Abbreviations: log K_{AW} = logarithmic air-water partition coefficient; log K_{OW} = logarithmic octanol-water partition coefficient.

Figure 1 Alt text: Scatter plot showing the chemical space of 53 petroleum hydrocarbons plotted by hydrophobicity and volatility. Green and blue points represent petroleum hydrocarbons, all located outside the shaded applicability domain of a 24-well polystyrene algal bioassay.

Figure 2. Overview of the literature review workflow combining two search strategies including the screening process and information extraction from peer-review literature. Steps where papers were included are indicated by blue arrows and the different steps, with resulting information included in this review, are indicated by green boxes.

Figure 2 Alt text: Flow diagram illustrating the literature review workflow, combining two search strategies followed by screening and information extraction. Blue arrows indicate inclusion steps, and green boxes show the stages that contributed data to the final review.

Figure 3. Heat map showing the number of studies identified in the literature search that evaluate substances with difficult to test properties (DTTP) relative to the reported dosing method and vessel type. The color gradient represents the number of papers reporting each combination of dosing, DTTP, and vessel category, with darker shading indicating higher study counts. The DTTP category “UVCB dttp NR” was used on biological products where hydrophobicity and/or volatility was not reported (NR). The vessel category “other/NR” includes studies where the vessel was not reported (NR), poorly

described, or did not fall into the predefined categories (e.g., flasks, slides, cuvettes). Figure created using JMP (2025). Abbreviations: DTTP = difficult-to-test properties; UVCB = substance of unknown or variable composition, complex reaction products or biological materials; NR = not reported; MAF = media accommodated fraction; WAF = water accommodated fraction.

Figure 3 Alt text: Heat map showing the number of literature studies assessing difficult-to-test substances across combinations of dosing method and test vessel type. Darker shading indicates more studies. Categories include UVCB substances and cases where volatility or hydrophobicity were not reported.

Figure 4. Principles of different dosing methods and proportions of papers with experiments using each method. Figure created in BioRender. Gomes T (2026) https://biorender.com/vb7u9rv. Abbreviations: DMSO = Dimethyl sulfoxide.

Figure 4 Alt text: Schematic diagram summarising major dosing methods used in the reviewed studies, alongside a chart showing the proportion of papers applying each dosing approach.

Figure 5. Chemical space of hydrocarbons (see Figure 1) overlapping the space of observed chemicals behaviour (shaded areas) determined from an experimental study in a 96-well microplate after incubation for 24 hr at 37°C (Birch et al. 2019). The dashed line represents a 'volatility cutoff' proposed based on an AREc32 cell assay in 384-well plates containing serum, above which chemicals are lost (Lee et al. 2022). The hatched lines represent an area of cross-over in 384-well plate assays (Lee et al. 2022). Abbreviations: log K_{AW} = logarithmic air-water partition coefficient; log K_{OW} = logarithmic octanol-water partition coefficient.

Figure 5 Alt text: Chemical space plot showing hydrocarbons positioned by hydrophobicity and volatility, overlaid with shaded regions representing experimentally observed chemical behaviour in microplate assays. A dashed line indicates a proposed volatility cutoff above which chemical losses occur, and hatched areas highlight crossover regions for 384-well plate assays.

Figure 6. Decision framework for selecting dosing approaches and interpreting in vitro tests with difficult-to-test substances and UVCBs. After problem formulation, systems minimizing losses and suitable dosing methods are selected. Analytical confirmation determines concentration stability, allowing responses to be linked to nominal or measured values when stable, or indicating when time-resolved concentrations may be derived. Abbreviations: HH= human health; ENV = environment; MoA = mode of action; GLP = Good Laboratory Practices; UVCB = substance of unknown or variable composition, complex reaction products or biological materials.

Figure 6 Alt text: Flowchart presenting a decision framework for selecting dosing methods and interpreting in vitro tests for difficult-to-test substances and UVCBs. The diagram includes steps for problem formulation, selection of exposure systems, analytical confirmation of concentration stability, and guidance on using nominal or measured concentrations.

Figure 7. Representations of the applicability domain of various dosing methods. The chemical space of the dosing methods (green boxes) is defined based on the hydrophobicity (x-axis) and the volatility (y-axis) of the whole UVCB test substance, where the lower left corner of the graph represents the water soluble, hydrophilic, non-volatile substances, and the upper right corner represent the highly volatile, highly hydrophobic substances. Any losses i.e., to volatilization or sorption after dosing, are not considered in this figure. Abbreviations: log K_{AW} = logarithmic air-water partition coefficient; log K_{OW} = logarithmic octanol-water partition coefficient; MAF = media accommodated fraction; WAF = water accommodated fraction.

Figure 7 Alt text: Conceptual chemical space diagram showing the applicability domains of different dosing methods as green boxes across axes of hydrophobicity and volatility.

Table 1. Keywords used in the literature search. Each of the search terms 1 in column 1 was combined with each of the search terms 2 in column 2 to give 33 search phrases that were used for both PubMed and Web of Science.

Search term 1	**Search term 2**
direct addition AND (petroleum OR multi-constituent OR multi-component)	in vitro techniques[mh] OR cell culture OR "in vitro" OR cell-based
solvent carrier AND (petroleum OR multi-constituent OR multi-component)	
passive dosing AND (petroleum OR multi-constituent OR multi-component)	
(media-accommodated fraction OR "media accommodated fraction" OR MAF) AND (petroleum OR multi-constituent OR multi-component)	mutagenicity tests OR mutagens OR mutagenicity OR mutagenic or "gene mutation" OR "ames " OR "comet assay"
(water-accommodated fraction OR "water accommodated fraction" OR WAF) AND (petroleum OR multi-constituent OR multi-component)	
passive dosing AND (hydrophobic)	
UVCB	(zebrafish AND embryo) or FET test
petroleum AND (volatile OR semivolatile)	
petroleum AND (hydrophobic OR superhydrophobic)	
petroleum AND (complex composition)	
(multi-constituent OR multi-component) AND (volatile or hydrophobic)	

Abbreviations: [mh] = MeSH (Medical Subject Headings), the National Library of Medicine controlled vocabulary thesaurus used for indexing articles in PubMed; FET = Fish Embryo Toxicity (test); MAF = media-accommodated fraction; UVCB = substances of unknown or variable composition, complex reaction products or biological materials; WAF = water-accommodated fraction

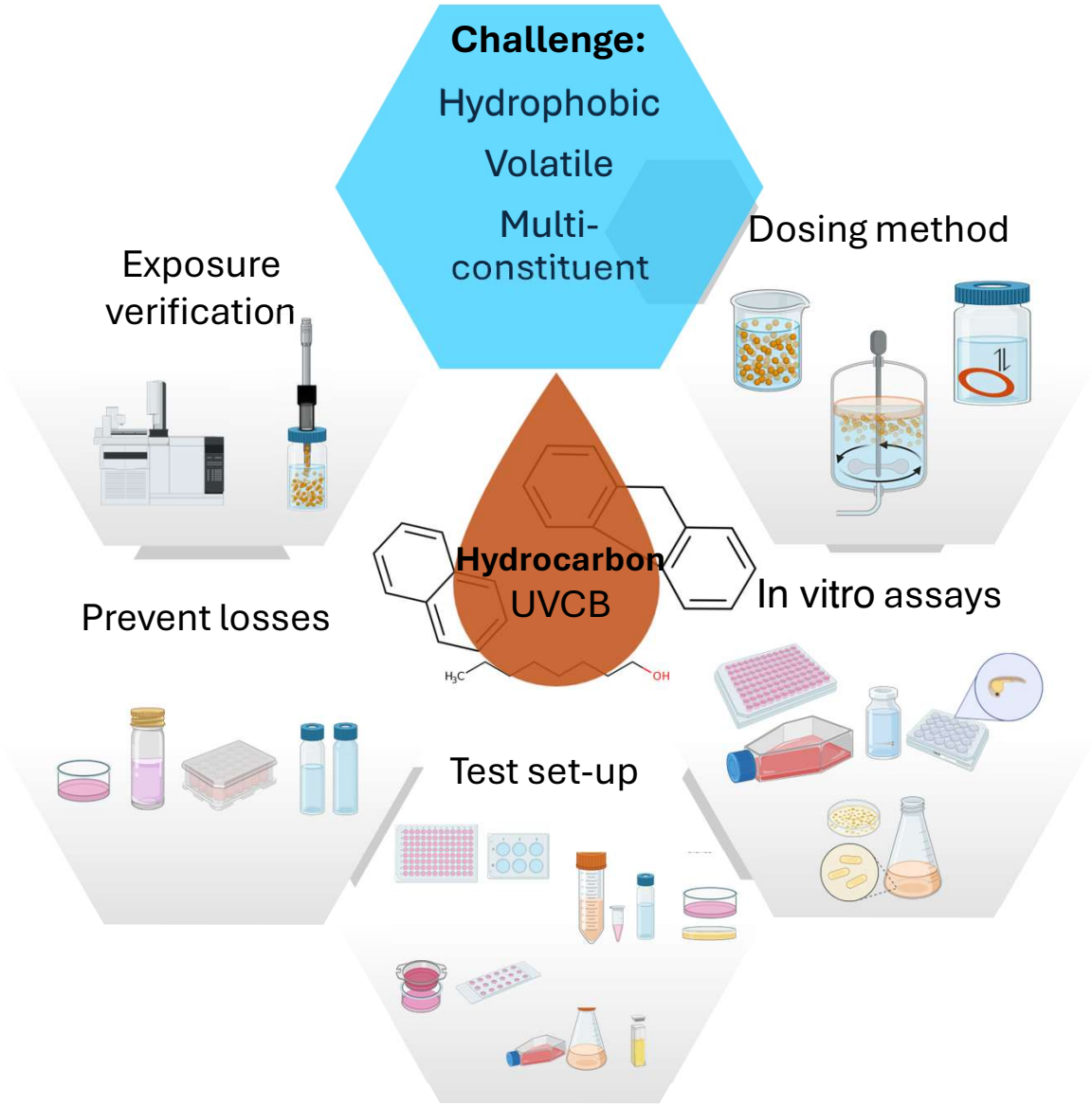
Table 2. Number of papers screened, excluded and included in the two literature search strategies.

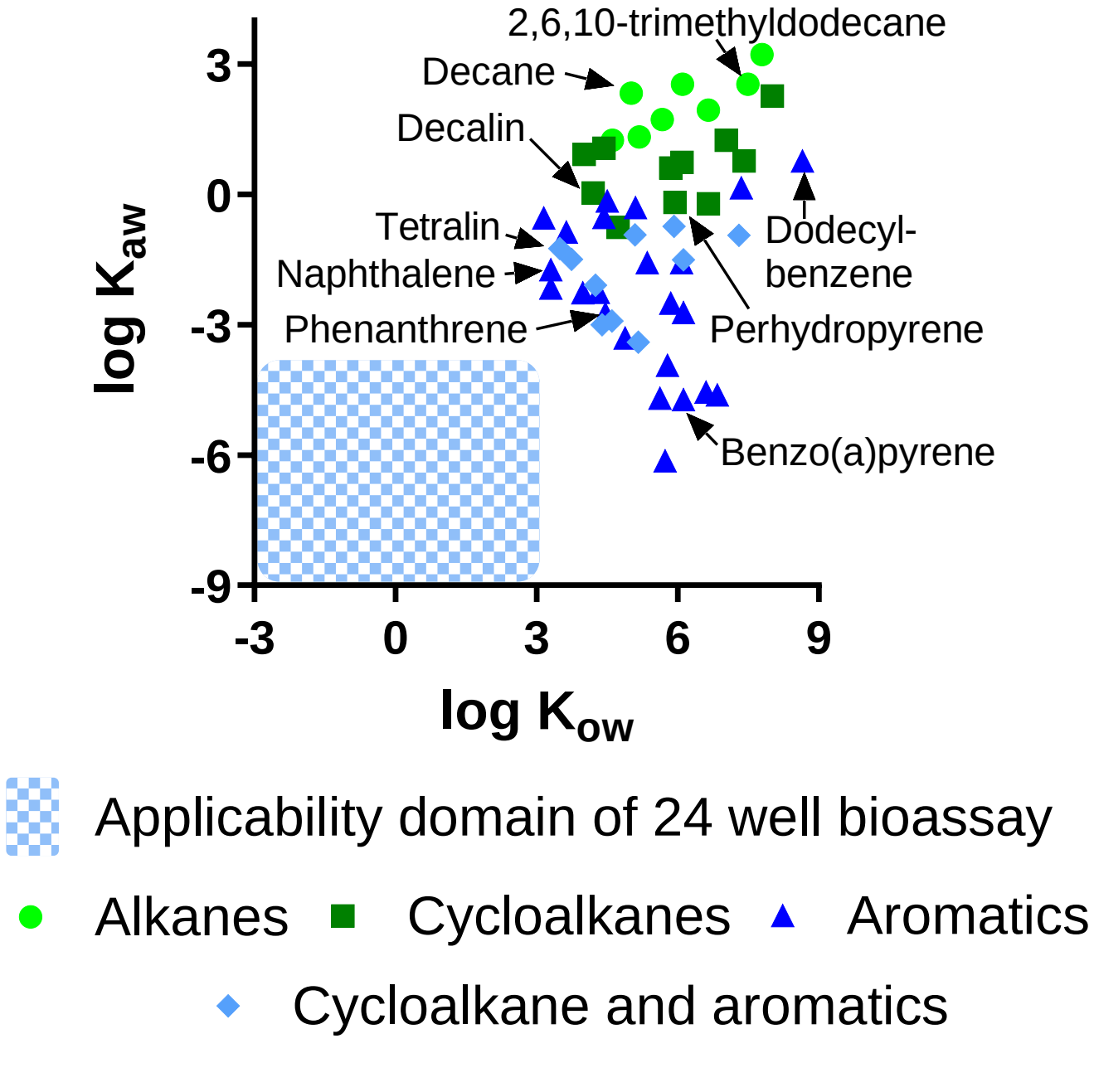
Search strategy	Keyword search	Targeted citation search
Input papers	4285 papers (3846 from PubMed, 452 from Web of Science)	411 papers (65 citations from Riedl and Altenburger [2007]; 107 from Heringa et al. [2004], 56 from Tanneberger et al. [2010], 108 from Smith et al. [2010a], 63 from Kramer et al. [2010], 12 citations and 43 references from Birch et al. [2019])
Screening process	Level 1 (Title and abstract): 3753 excluded, 532 included	**150 papers included**
	Level 2 (Materials & methods): 303 excluded, **225 included**	
Experimental papers	188 from keyword search including 193 studies, 44 from citation search including 53 studies, 12 papers covered in both searches including 18 studies	

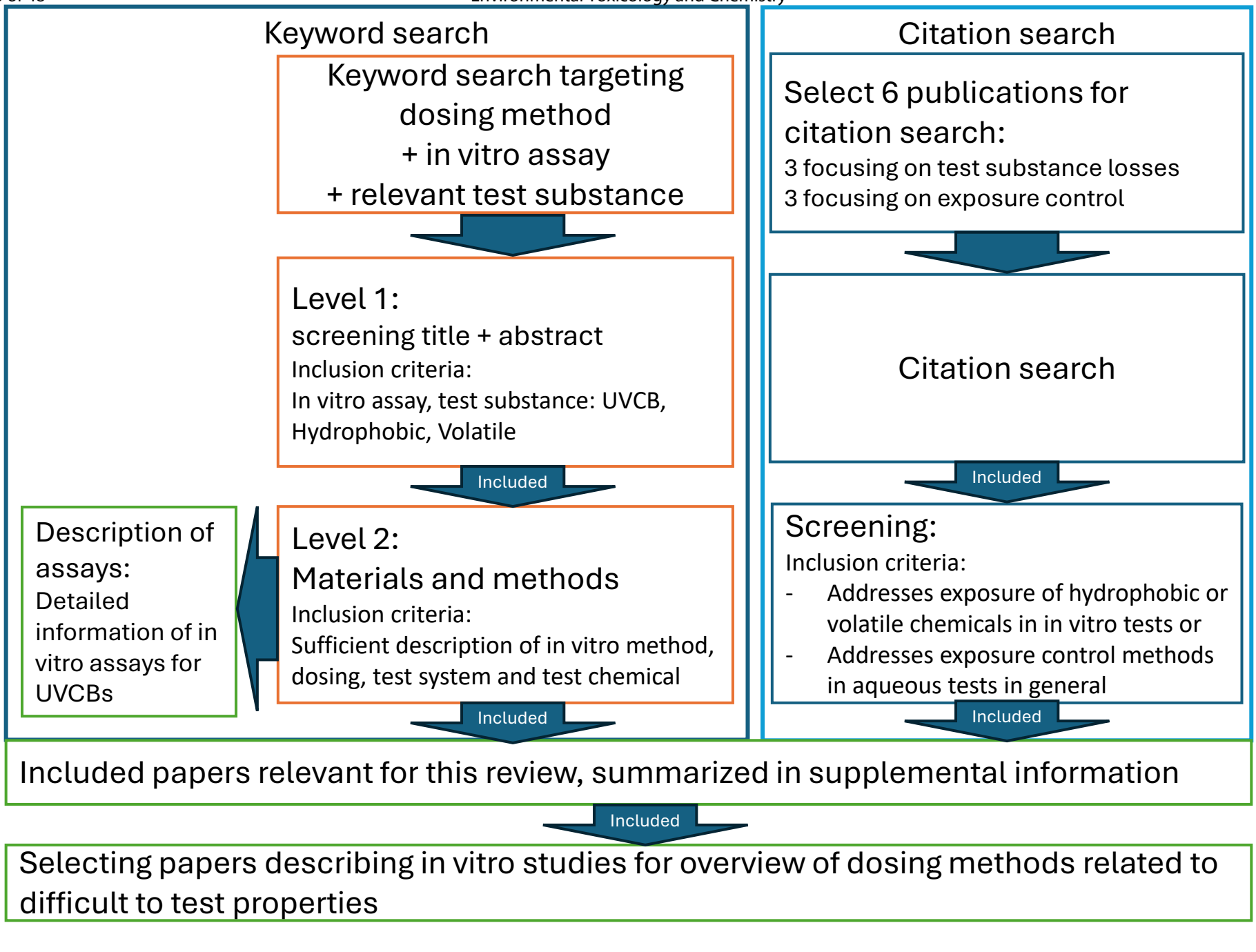
Table 3. Advantages and disadvantages of different in vitro dosing methods.

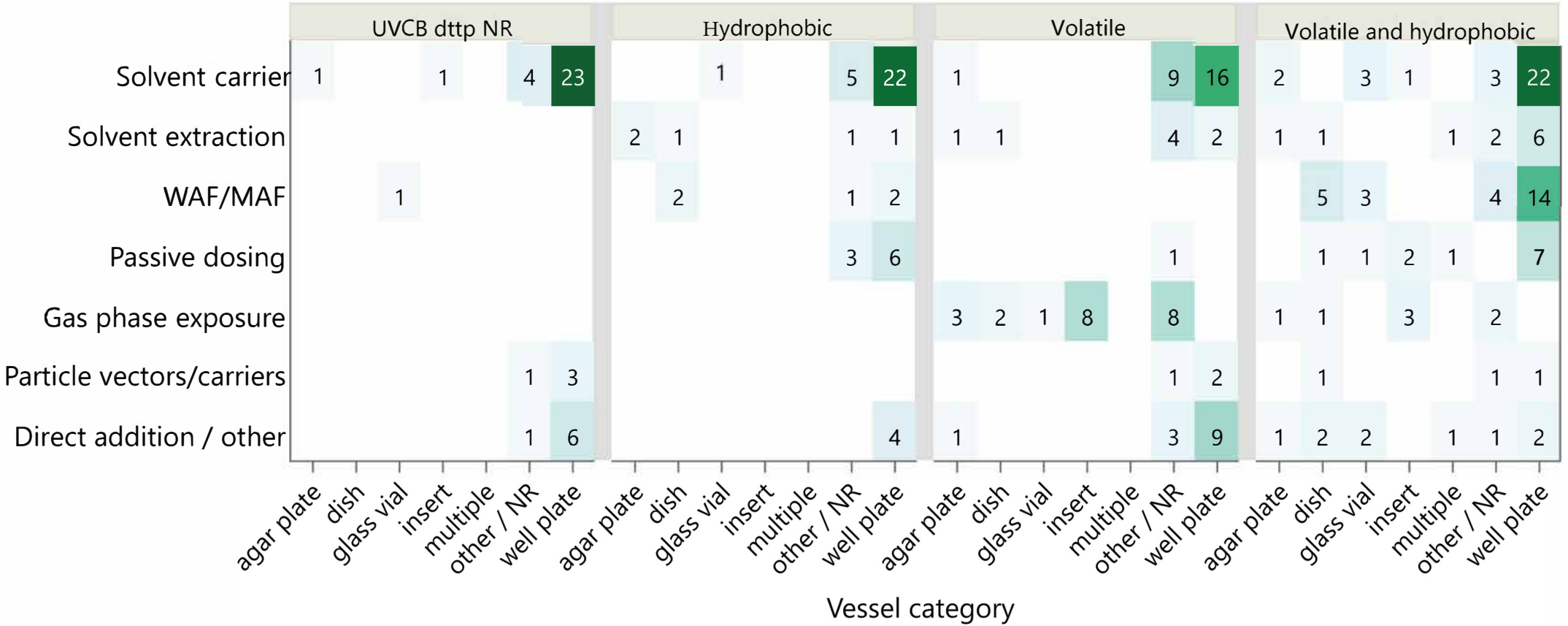
Dosing method	Compound Properties	Advantages	Disadvantages
Solvent carrier	Hydrophobic chemicals (log K_{OW}>3)	Whole substance dosing; Simple to prepare	Solvent might affect medium components or test system; Non-homogeneous exposure if spiked directly or above solubility
Solvent extraction	Hydrophobic substances	Represents only the fraction of the UVCB that is extracted into solvent.	Choice of solvent alters composition and outcome; Observed toxicity may not reflect toxicity of whole substance (see also solvent carrier).
WAF/MAF	UVCBs with constituents spanning a range of hydrophobicity (especially log K_{OW} < 3–4)	Relatively simple to prepare; avoids co-solvent effects; Serum can stabilize free concentrations of reversibly bound constituents; Simulates environmentally relevant testing of UVCBs (i.e., preparation of HE WAFs can reflect spill scenarios, whereas LE WAFs are commonly prepared in regulatory toxicity testing).	Some constituents may bind to organic material (e.g., serum) in the MAF reducing bioavailability; Not suitable for volatile substances; Does not stabilize concentrations.
Passive dosing	Hydrophobic chemicals with log K_{OW} > 3; (semi)volatile chemicals	Maintains stable concentrations and composition; Supports well-defined concentration-response testing; Avoids dosing above solubility; Avoids co-solvent effects; Can simulate spill scenarios where undissolved droplets function as reservoirs to maintain dissolved concentrations in water.	Loading of dosing phase is time-consuming; Requires understanding of partitioning principles and some level of experience to avoid pitfalls; Difficult to apply to ≥96 well plates.
Particle carriers	Hydrophobic, (semi)volatile, easily degradable	Increased compound stability; Controlled release; Increased efficiency compared to pure compound.	Optimal delivery depends on carrier type and substance properties; Uncertainties on aspects controlling efficiency; Limited validation, so method development needed.
Gas-phase generator exposure systems	Gaseous, (semi)volatile, particulate	Minimizes volatilization losses; Reduces reactions with medium components (particularly at ALI); Enables more realistic exposure conditions (in case of ALI); Possibility to separate gas and particle phases; Permits real-time monitoring of exposure.	Suitability dependent on system design and air-flow configuration; Certain flow system set-ups may not be appropriate for all volatile substances (e.g., perpendicular flow not suitable for gasoline); Not suitable for chemicals with very high or low vapor pressures or that are highly hygroscopic or polymerizable.

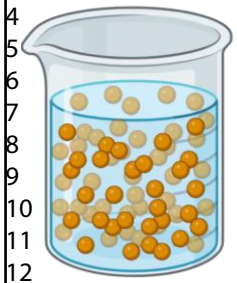
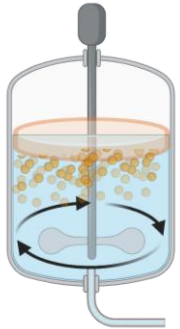
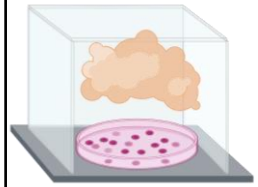
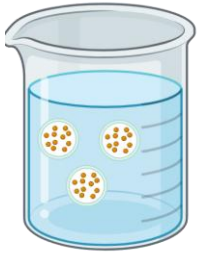
Abbreviations: ALI = air–liquid interface; HE = high energy; LE = low energy; log K_{OW} = logarithmic octanol–water partition coefficient; MAF = media accommodated fraction; UVCB = substance of unknown or variable composition, complex reaction products or biological materials; WAF = water accommodated fraction

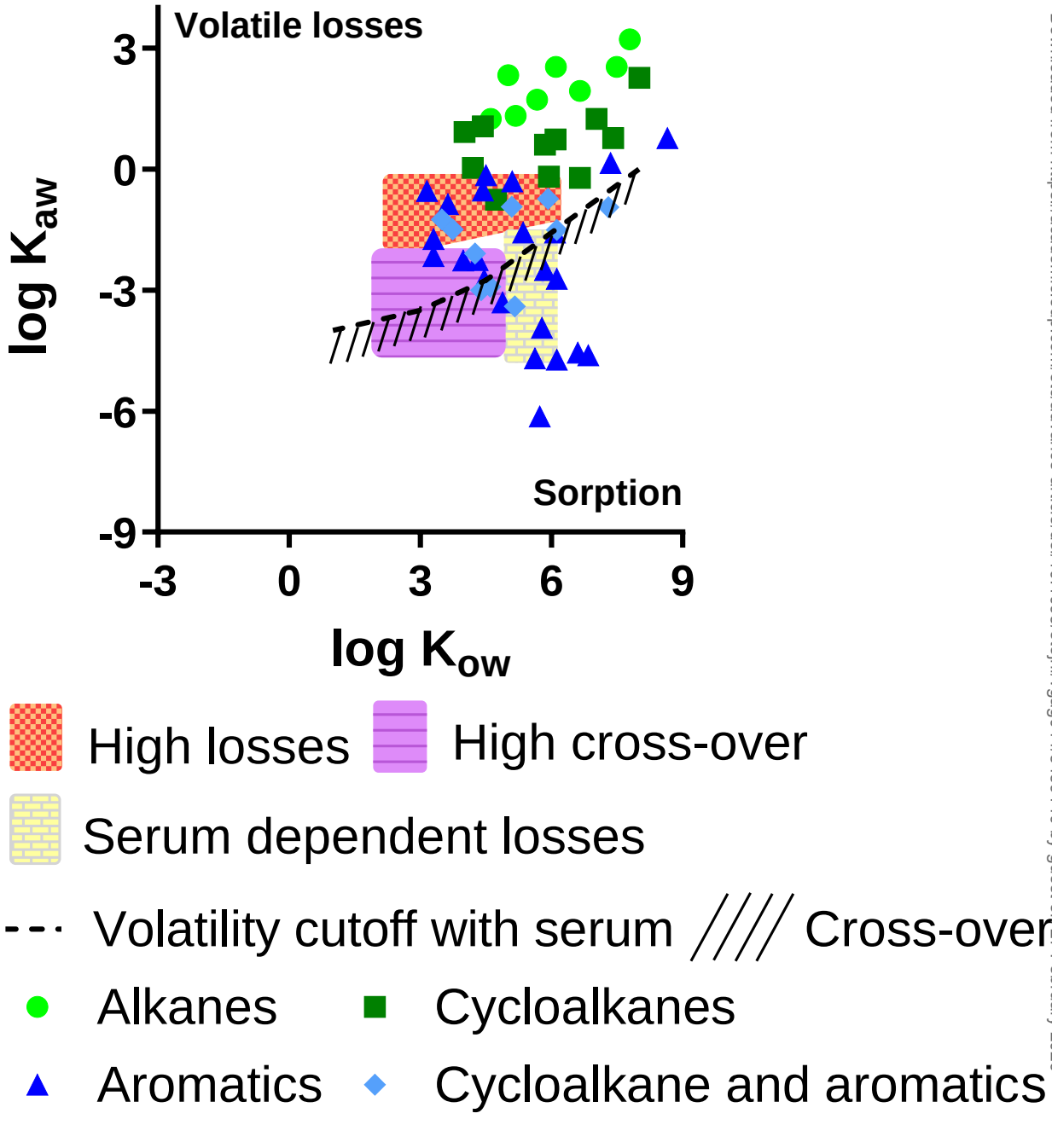








Solvent carrier	Solvent extraction	Media or water accommodated fractions	Passive dosing	Gas phase exposure	Dosing with particle carriers
					
Dissolve substance in water miscible solvent	Extract substance with solvent (e.g., DMSO) and spike extract	Stir substance and allow to separate and use medium for testing	Establish and maintain constant exposure via partitioning from loaded polymer	Dose volatile substance via gas phase in a closed or flow through system	Targeted dosing of substances with particulate vectors or nano carriers
43 % (114 studies)	9.1 % (24 studies)	12 % (32 studies)	8.3 % (22 studies)	11 % (29 studies)	3.8 % (10 studies)



Problem formulation

- ***Define purpose and decision context***
  - Examples: HH/ENV hazard or risk assessments; read-across; MoA determination
- ***Define exposure route and dosing approach***
  - Align dosing with exposure route and duration (inhalation, ingestion, dermal; acute vs repeated)
- ***Identify key biological or toxicological endpoints***
- ***Note relevant chemical properties and substance characteristics***
  - e.g., hydrophobicity, volatility, UVCB
- ***Note possible constraints on method adaptation***
  - If the test is for regulatory purposes, there may be less flexibility for test modifications in order to comply with relevant test guidelines and GLP

Test set-up

- Select a test system that minimizes evaporative and sorptive losses
- Select a dosing method that reflects exposure and accounts for the properties of the test substance

Analytical confirmation

Does the substance composition and test concentrations remain stable during the study?

No

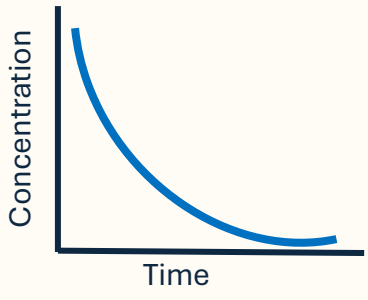
Yes

Modify approach

Can actions be taken to stabilize test concentrations (e.g., repeated dosing, static renewals, passive dosing, flow through test design)?

No

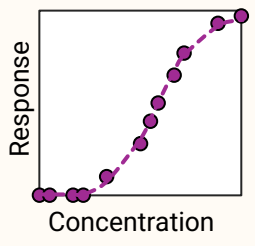
Combine analytical measurements with predictive approaches to determine time resolved test concentrations



Yes

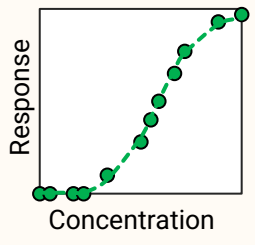
Conduct tests

Relate response to ***nominal or measured*** concentrations



Yes

Relate response to ***controlled or measured*** concentrations



Yes

Relate response to ***measured or modelled*** concentrations