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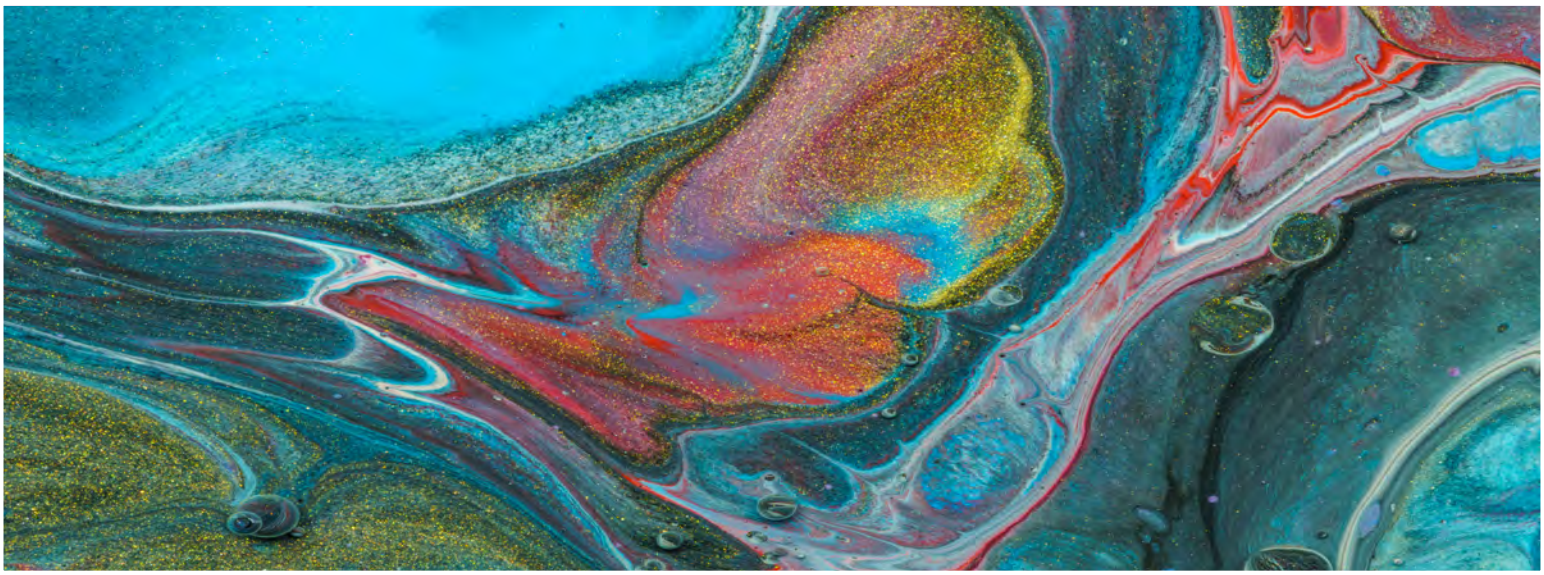
Advances in Dose Addition for Chemical Mixtures: A White Paper



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Advances in Dose Addition for Chemical Mixtures: A White Paper

Risk Assessment Forum
Cumulative Risk Assessment Technical Panel

Risk Assessment Forum
U.S. Environmental Protection Agency
Washington, DC 20460

DISCLAIMER

This document provides a summary of the state of the science on advances in dose addition for chemical mixtures. While this document reflects U.S. EPA's summary of the best available science, it is not a regulation and does not impose legally binding requirements on U.S. EPA, states, Tribes, or the regulated community, and might not apply to a particular situation based upon the circumstances. It does not represent and should not be construed to represent any Agency determination or policy. U.S. EPA may change this document in the future. This document has undergone contractor-led external peer review as well as a review process within U.S. EPA. Final review by U.S. EPA's Science and Technology Policy Council has been completed and the document has been approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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ACRONYMS AND ABBREVIATIONS

ADME	absorption, distribution, metabolism, and excretion
AEGL	acute exposure guideline level
AhR	aryl hydrocarbon receptor
AO	adverse outcome
AOP	adverse outcome pathway
ARE	acute reference exposure
As	arsenic
ATSDR	Agency for Toxic Substances and Disease Registry
BDCM	bromodichloromethane
b_i, B_i	dose coefficients
BMDS	Benchmark Dose Software
Cd	cadmium
CERCLA	Comprehensive Environmental Response, Compensation, and Liability Act
$CHBr_3$	bromoform
CMG	common mechanism group
Cr	chromium
Cu	copper
DBCM	chlorodibromomethane
DBP	disinfection by-product
DCA	dichloroacetic acid
d_i	component dose
DLC	dioxin-like compound
D_{mix}	total mixture dose
DNA	deoxyribonucleic acid
DR	dose response
DRC	dose-response curve
E	exposure
ECHA	European Chemicals Agency
ED	effective dose
EROD	ethoxyresorufin-O-deethylase
FQPA	Food Quality Protection Act
GCA	generalized concentration addition
GM	geometric mean
GTP	guanosine-5′-triphosphate

HA	Health Advisory
HI	hazard index
HI_{INT}	interaction-based HI
HQ	hazard quotient
HTP	high throughput
IATA	Integrated Approach to Testing and Assessment
IC	index chemical
ICED	index chemical-equivalent dose
$ICED_{MIX}$	index chemical-equivalent dose of a mixture
IRIS	Integrated Risk Information System
IVIVE	in vitro to in vivo extrapolation
KE	key event
KER	key event relationship
LOAEL	lowest-observed-adverse-effect level
LOEL	lowest-observed-effect level
LOTEL	lowest-observed-transcriptional-effect level
MHI	multiroute/multipathway hazard index
MIE	molecular initiating event
MLE	maximum-likelihood estimate
Mn	manganese
MOA	mode of action
MOE	margin of exposure
MOE_T	total margin of exposure
MRL	minimal risk level
NAMs	new approach methodologies
NAS	National Academy of Sciences
NF-κB	nuclear factor kappa b
NOAEL	no-observed-adverse-effect level
NRC	National Research Council
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
OPP	Office of Pesticide Programs
Pax8	paired box 8
Pb	lead
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin

PCDF	polychlorinated dibenzofuran
p_i	fractional component dose
PKCK2	protein kinase casein kinase 2
p_{mix}	probability of response to the chemical mixture
POD	point of departure
p_x	component risk
RAF	Risk Assessment Forum
RAGS	Risk Assessment Guidance for Superfund
ReP	relative potency
RfC	reference concentration
RfD	reference dose
RfV	reference value
r_{mix}	total mixture risk of a common effect
RPF	relative potency factor
SARA	Superfund Amendments and Reauthorization Act
SF	slope factor
Smad 2,4	mothers against decapentaplegic homolog 2 and 4
SMRT	silencing mediator of retinoic acid and thyroid hormone receptor
T3	triiodothyronine
T4	thyroxine
TCA	trichloroacetic acid
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TD	toxicodynamic
TEF	toxicity equivalency factor
TGF-β1R	transforming growth factor beta-1 receptor
TK	toxicokinetic
TNF-α	tumor necrosis factor alpha
TOSHI	target organ-specific hazard index
TP	toxicity pathway
TPO	thyroid peroxidase
TSCA	Toxic Substances Control Act
TSH	thyroid stimulating hormone
TTD	target-organ toxicity dose
TTF-1	thyroid transcription factor 1
U.S. EPA	U.S. Environmental Protection Agency
UDPGT	uridine 5′-diphospho-glucuronosyltransferase

UF	uncertainty factor
WHO	World Health Organization
WOE	weight of evidence
WP	Weibull power
Zn	zinc

FOREWORD

This White Paper describes current U.S. Environmental Protection Agency (U.S. EPA) practices associated with dose-additive methods for human health risk assessments of chemical mixtures. It discusses challenges associated with the use of toxicological information and individual chemical dose-response information when applying dose-additive methods and considers the potential for future applications of dose addition that take advantage of kinetic and dynamic data generated from new approach methodologies (NAMs).

These dose-additive methods follow U.S. EPA's 1986 *Guidelines for the Health Risk Assessment of Chemical Mixtures* (U.S. EPA 1986) and U.S. EPA's 2000 *Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (U.S. EPA 2000c). These two documents describe several dose-additive methods that were available for use by U.S. EPA to assess health risks from chemical mixtures, and also stress the feasibility, practicality, and usefulness of these methods.

This document has been developed to advance cumulative risk assessment, specifically chemical mixtures risk assessment within the broad field of cumulative risk assessment, informed by U.S. EPA's experience and scientific progress since 2000. It responds to the following: (1) U.S. EPA's experience applying dose-additive methods in human health risk assessments of environmental chemical mixtures; (2) publications of methods based on dose addition by U.S. EPA's Office of Research and Development and U.S. EPA's programs, offices, and regions; (3) publications in the scientific literature since 2000 that are related to dose addition; and (4) advances in the fields of experimental design, biomathematics, toxicology, molecular and cellular biology, and biochemistry that have augmented and improved the scientific understanding of biological responses to chemicals at different levels of biological organization.

This document reviews current uses of dose addition in U.S. EPA mixture assessments and discusses possible avenues of research and development for consideration in future mixture risk assessment practice. This document includes presentation, clarification, and discussion of the following:

- Current practices and methods within U.S. EPA that are based on dose addition and that address health risks and health hazards posed by exposures to mixtures of chemicals;
- Considerations for grouping chemicals when applying methods based on dose addition;

- Expansion of methods for the prediction of chemical mixture risks based on dose addition; and
- Potential integration of data generated through NAM studies in mixture risk assessment methods based on dose addition.

This document was prepared by a Risk Assessment Forum (RAF) Cumulative Risk Assessment Technical Panel composed of senior risk assessors and toxicologists from across U.S. EPA and through an interagency agreement with Argonne National Laboratories and a U.S. EPA contract. All activities were overseen by the RAF. The RAF is a standing committee of senior U.S. EPA scientists that was established to promote Agency-wide consensus on risk assessment issues and to ensure that this consensus is incorporated into appropriate U.S. EPA risk assessment guidance. The purpose of this panel is to advance the application of cumulative risk assessment to inform U.S. EPA risk management decisions.

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DEDICATION

This White Paper is dedicated to the memories of our co-authors, Mr. Jeffrey Swartout and Dr. Jane Ellen Simmons, who sadly passed away during the writing of this document. The following loving tributes attempt to capture and describe their creative spirits and remarkable contributions to this White Paper and, ultimately, to the field of chemical mixtures risk assessment. In addition to being gifted scientists, both Jeff and Jane Ellen were cherished friends who are deeply missed.

Jeff, a toxicologist and risk assessment scientist, worked for the U.S. Environmental Protection Agency's Office of Research and Development for over 37 years. A mainstay of the Agency's work in human health risk assessment, he was an expert in the development of dose-response assessments for health effects following exposures to chemicals, with particular emphasis on quantitative uncertainty analysis. Jeff's contributions to chemical mixtures risk assessment included theoretical improvements to dose-addition-based methods, in particular, the application of variable relative potency factors. He continually championed the benefits of improved data, especially from cutting edge studies, and demonstrated practical solutions to complex issues that other researchers can use to further advance mixtures risk assessment. Jeff was a brilliant, creative, and insightful risk assessor and colleague. Among the many qualities that made him an outstanding risk assessor, Jeff truly loved the dogged challenge of pursuing "the best answer" to a dose-response assessment or uncertainty analysis question. He was a fantastic collaborator in the risk assessment community because of his ability to evaluate complex issues and scientific data through different lenses, his exceptional work ethic, his quick wit, and the sincere joy that he brought to any endeavor.

Jane Ellen, a toxicologist and research scientist, exhibited a deep and irrepressible passion for the rigorous scientific pursuit of understanding how human health can be affected by exposure to mixtures of chemicals in the environment. Jane Ellen was a scientist's scientist. She was a top-notch laboratory toxicologist at the U.S. Environmental Protection Agency's Office of Research and Development for 36 years, internationally known expert on chemical mixtures, superb liaison across researchers, coordinator and leader of multidisciplinary projects, and amazing presenter and author of complex information. She was an internationally recognized expert on chemical mixtures toxicology and a frequent sponsor of, and collaborator with, quantitative risk assessment researchers. She continually pushed the Agency to generate toxicity data useful for environmental health risk assessment. Jane Ellen contributed significantly to formulating and refining foundational principles of chemical mixtures toxicology and risk assessment by focusing on optimization of study design and results generation that informed regulatory decision making. Jane Ellen brought joy daily to the research projects she pursued. Her enthusiasm for contributing "good science" was boundless.

EXECUTIVE SUMMARY OF ADVANCES IN DOSE ADDITION FOR CHEMICAL MIXTURES: A WHITE PAPER

Many human exposures involve concurrent or temporal sequential exposures to mixtures of chemicals; as such, the complexity of assessing joint toxicity associated with multichemical exposures warrants continued advancement of methods and approaches. Multiple laws direct the U.S. Environmental Protection Agency (U.S. EPA) to address health risks posed by exposures to chemical mixtures, such as the Toxic Substances Control Act of 1976 (amended 2016). Methods for evaluating the health risks of chemical mixtures were developed primarily based on how chemicals act when co-occurring in biological matrices (e.g., target organs/tissues).

The intent of this White Paper is to explain methods currently in use by U.S. EPA and to stimulate development of improved methods for assessing health risks from environmental chemical mixtures by discussing advances in practices for evaluating chemical mixtures and considerations when applying new sources of toxicological data. This White Paper describes current U.S. EPA practices associated with dose-additive methods for human health risk assessments of chemical mixtures encountered in the environment. In particular, it:

- addresses challenges associated with the use of toxicological information and individual chemical dose-response information when applying such methods,
- considers the potential for future applications of dose addition that use toxicokinetic and toxicodynamic data generated from new approach methodologies (NAMs), and
- discusses uncertainties in conducting risk and hazard assessments using dose-additive mixture methods.

These dose-additive methods are consistent with U.S. EPA's 1986 *Guidelines for the Health Risk Assessment of Chemical Mixtures* (U.S. EPA 1986) and U.S. EPA's 2000 *Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (U.S. EPA 2000c). Dose addition provides a simple mathematical approach for estimating joint toxicity associated with complex interactions among biological systems and combinations of chemicals encountered in the environment. The use of dose-additive models is the default approach of U.S. EPA for assessing noncancer health hazards from mixtures of toxicologically similar chemicals because it has been shown to be, for similar chemicals, reasonably predictive of combined effects (U.S. EPA 2000c). Dose-additive models also are used to estimate cancer risks when the chemicals are similar in terms of carcinogenicity (e.g., U.S. EPA 1993; 2010).

This White Paper considers the following: (1) U.S.EPA's experience applying dose-additive methods in human health risk assessments; (2) publications of methods based on dose addition by U.S. EPA; (3) publications in the scientific literature since 2000 that are related

to dose addition; and (4) advances in the fields of experimental design, biomathematics, toxicology, molecular and cellular biology, and biochemistry that have improved the scientific understanding of responses to chemicals at different levels of biological organization.

A Risk Assessment Forum (RAF) Cumulative Risk Assessment Technical Panel prepared this White Paper to advance chemical mixtures risk assessment within the broader field of cumulative risk assessment. The goal of the RAF "is to address risk assessment issues and develop Agency-wide guidelines, guidance and methods in support of Agency decision making in its mission to protect human health and the environment" (U.S. EPA 2016). U.S. EPA (2003c) defines cumulative risk assessment as "an analysis, characterization, and possible quantification of the combined risks to health… from multiple agents…." Chemical mixtures risk assessment is a type of cumulative assessment in which all agents are chemicals.

Section 1 describes the purpose and organization of this document. It clarifies the scope and provides context with a brief history of U.S. EPA applications of dose-additive methods to mixtures of chemicals encountered in the environment. This document's intended audience includes risk assessors and risk managers who participate in Agency evaluations of human health risks posed by chemical mixtures, particularly those involving dose-additive methods. Research toxicologists studying chemical mixtures also may find this document useful.

This White Paper does not directly address assessment of ecological risk, nor analyses of human epidemiological data. It does not address whole mixtures nor does it consider comparisons of responses predicted on the basis of dose-additive models to observed responses to whole mixtures (i.e., validation experiments). The document is not intended to serve as a repository for or analysis of previous U.S. EPA mixture assessments that relied on dose-additive models. This White Paper also does not focus on the many exposure assessment issues that can arise during the conduct of chemical mixtures risk assessments.

Section 2 of the White Paper explains how the consideration of joint toxic action differentiates human health risk assessment of a mixture of chemicals from single chemical assessments. The term "toxicological interaction" is described as a deviation from the mixture risk or hazard predicted using a dose-additive method (e.g., synergy; antagonism).

Two main concepts underpin U.S. EPA's guidance on component-based mixture approaches: simple similar action (Bliss 1939) and simple independent action (Finney 1971).

Simple similar action applies to chemicals that are toxicologically similar and cause a common health effect. Simple similar action means that chemicals act as if they are dilutions or concentrations of each other, eliciting the common effect by the same mechanism of action (U.S. EPA 2000c). Methods based on dose addition generally are applied when assuming that chemicals act through simple similar action.

Simple independent action applies to toxicologically dissimilar chemicals that cause a common health effect, where "dissimilar" is defined as chemicals having different toxic mechanisms. This means that the responses to different chemicals are independent events (U.S. EPA 2000c). Response addition is generally applied when it is established that chemicals are toxicologically dissimilar.

Dose addition is the approach used by U.S. EPA to predict the response to a mixture under conditions of simple similar action. Historically, this has been interpreted by thinking of the mixture as a single chemical solution comprised of various dilutions of the same chemical (Bliss 1939), an interpretation some call the Finney definition because of the author's early publications (Finney 1942). Mixture risk methods used by U.S. EPA have followed the Finney definition. A more general definition of dose addition that does not assume constant relative potency was described by Berenbaum (1977; 1985; 1989). (See **Section 4**.)

Response addition includes the approaches used to predict the response to a mixture under conditions of simple independent action. Under such conditions, the mixture chemicals cause the same specific or general effect, and some measure of the toxic impact of each chemical is summed. Two types of mixture risk assessment methods are based on simple independent action: response addition and effect summation. The response addition method uses toxicity measured as the probability (risk) of the health effect of concern. The response to the mixture is predicted by summing the risk estimates for the mixture components under the law of statistical independence. In effect summation, the biological measurements associated with the individual mixture component doses are added (e.g., sum the incremental changes in diastolic blood pressure caused by each of the mixture components). Although rarely employed in human health risk assessment, effect summation is usually restricted to small incremental changes.

The primary criterion for choosing between dose addition and response addition methods is toxicological similarity among the chemicals in the mixture (U.S. EPA 2000c). "Toxicological similarity" is used here as an overarching concept with a wide range of specificity across levels of biological organization, allowing similarity judgments to be tailored to both the specific goals of the mixture risk assessment and the availability of hazard and dose-response information across components. The concept of "similarity of toxic action" is more focused, usually relating to pathway-based key events (KEs) or toxic mechanisms.

The information used to inform toxicological similarity can vary widely in type and availability across the chemicals comprising a mixture of interest. Data that could potentially inform similarity in toxic action can be collected through studies conducted at different levels of biological organization (e.g., subcellular, tissue, organ), through different types of studies (e.g., in vitro or in vivo), across different test species or human populations. Alternatively, similarity among mixture chemicals could be predicted or inferred based upon shared structural

similarity(-ies), physicochemical properties, and/or absorption, distribution, metabolism, and elimination characteristics. Several publications pertaining to similarity in toxic action focus on structured toxicodynamics, using conceptual terms such as mode of action (MOA), mechanism of action, toxicity pathways, and adverse outcome pathways (AOPs) (U.S. EPA 2017). These toxicodynamic concepts and several examples are further discussed in **Section 3.5**. Characteristics useful for determining similarity of toxic action among mixture component chemicals (including toxicokinetic characteristics) are addressed in **Section 3.2**.

Section 2 also describes current practices and methods within U.S. EPA that are based on dose addition. Dose-additive methods include hazard index (HI) approaches and variants, relative potency factor (RPF) approaches, and integrated addition approaches (Figure ES-1).

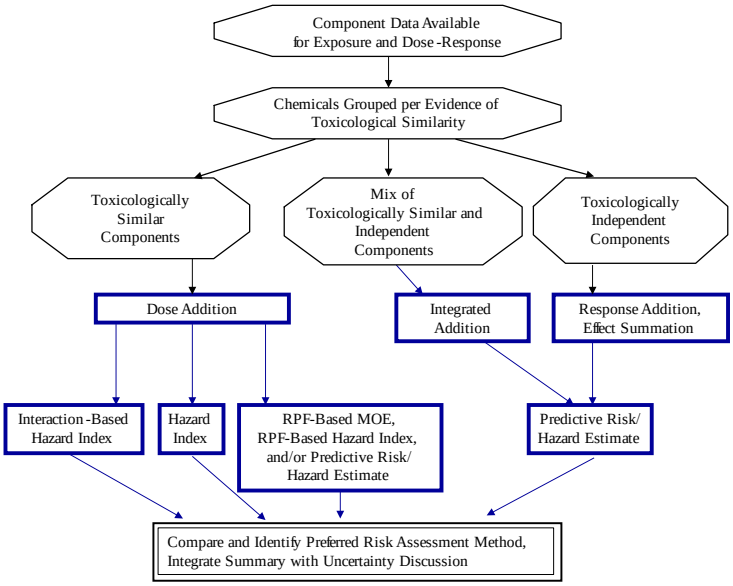


Figure ES-1. Flow chart for evaluating chemical mixtures using additive methods.

Path selected is based on strength of the relevant evidence for toxicological similarity and toxicological independence. Rectangles indicate specific component-based assessment methods; octagons indicate results of data gathering or evaluation.
MOE = margin of exposure; RPF = relative potency factor.

HI approaches are among the most widely used component-based mixture risk assessment methods. The four HI variants described herein are applied in the risk characterization step of a mixture risk assessment; they are decision aids for which a value exceeding 1 indicates the potential for toxicological hazard. In these HI formulas, the calculation is the sum of component-based fractions, each comparing the estimated population exposure (the

numerator) to a health hazard-based reference value (RfV; the denominator); the sum characterizes the potential for adverse health effects associated with the chemical mixture.

- **Hazard Index** Among the HI variants, this approach includes all of the component chemicals regardless of the critical effect domain on which each individual RfV is based.
- **Target Organ-Based Hazard Index** assumes all of the mixture chemicals in the assessment group cause a common profile of effect(s), and each potency weighting factor (1/RfV) reflects toxic potency for that chemical within a specific effect domain (e.g., the critical effect for each toxicity value across mixture components occurs in the liver).
- **Multiroute Hazard Index (MHI)** addresses environmental exposures involving more than one exposure route or pathway. The MHI is calculated by summing HIs across all exposure pathways using route-specific RfVs (U.S. EPA 1991a).
- **Interaction-Based Hazard Index (HI_{INT})** can incorporate known information on pairwise toxicological interactions. The HI, target organ-based HI, and MHI assume no toxicological interactions.

RPF approaches comprise the second basic dose addition method used by U.S. EPA. There are two types: the general RPF approach that has been applied to pesticides and a few other chemical groups, and the toxicity equivalency factor (TEF) approach, considered a special case of the RPF approach originally developed for mixtures of dioxins and dioxin-like compounds.

In the RPF approaches, a numerical quantity is used to scale the dose of one chemical to an equitoxic dose of another chemical (i.e., the index chemical) by accounting for differences in their potencies in causing the same/similar health effect. The index chemical, usually the chemical with the highest quality toxicological database in the mixture and the chemical considered to be most representative of the type of toxicity caused by the other mixture components (U.S. EPA 2000c; 2002a; 2002b), must have dose-response data for the dose range of interest. The RPF is the ratio of the potency of the individual component to that of the index chemical. The products of the individual chemical RPFs and the individual chemical exposure rates are then summed to yield an index chemical-equivalent dose (ICED) for the mixture. This ICED is then interpreted using the index chemical's dose-response curve (DRC) to characterize the risk or hazard, often by comparing to the cancer risk decision point for the scenario assessed (e.g., 10E-6) or to the noncancer reference dose/concentration of the index chemical.

Relative to TEFs, the health endpoints addressed by RPF applications may be more restricted, e.g., to specific health outcomes, specific exposure routes, specific exposure durations,

and/or a limited dose range. These restrictions of RPF applications are based on the underlying toxicological database of the mixture components. To date, TEF applications generally have been underpinned by more abundant and higher quality toxicological information when compared to RPF applications.

U.S. EPA developed the integrated addition approach for mixture exposures containing component chemicals that are not toxicologically similar but affect the same health endpoint. The approach incorporates both dose addition and response addition for toxicity endpoints, producing a probabilistic risk estimate of the adverse endpoint of concern for the mixture (Teuschler et al. 2004; U.S. EPA 2003b). The integrated addition approach initially separates the mixture components into dose-additive groups based on toxicological similarity. Next, the assumptions of similarity within groups and then of independence across groups are evaluated by examining existing mixture studies for evidence of interactions. If toxicological similarity is indicated for the chemicals within each dose-additive group, the RPF approach is used to estimate separately the risk for each dose-additive group. Individual group risks are then combined using response addition.

Section 3 identifies considerations that can be useful when evaluating whether potentially relevant chemicals could be included in a dose-additive group. Determining which environmental chemicals to include or exclude from the similarity group can be a major source of uncertainty during mixture assessment.

Dose addition is applied both across a broad spectrum of chemical mixtures, for which the levels of toxicity and exposure information among the individual component chemicals can vary widely, and across many different situations where chemical mixtures could be encountered in the environment. These considerations are intended to encompass this broad spectrum of potential applications. Application of these considerations also depends on the specific statutory, program-specific, or office-specific requirements prompting the risk assessment.

Depending on the U.S. EPA program or office and the purpose of the mixture risk assessment, two major considerations are used by U.S. EPA when evaluating component chemicals for possible inclusion in the dose-additive group: overlapping exposures and toxicological similarity.

ENVIRONMENTAL EXPOSURE TO MIXTURES

For dose addition to apply, environmental exposure to the component chemicals would need to occur within a time frame that results in the overlap of internal doses of the chemicals. These internal doses could represent parent mixture component chemicals to which individuals are exposed as well as associated bioactive metabolites (i.e., those doses of the chemicals need to be present together for dose addition to apply). For example, if chemicals occur in the same

environmental medium (e.g., food products) at the same time and if people contact the medium (e.g., food consumption), then it could be reasonably assumed that the doses of the chemicals would overlap in time.

SIMILARITY OF TOXIC ACTION

Confidence in assessments that rely on dose-additive methods is strengthened if some degree of similarity in toxicokinetic and/or toxicodynamic action is demonstrated among the chemicals in the mixture being evaluated.

The evidence for similar toxic action can be listed along a continuum. From most to least informative based on similarity, a plausible listing of the evidence is as follows:

a) Same toxic action: Same molecular initiating event (MIE) and subsequent downstream biochemical and biophysical processes (kinetic and/or dynamic KEs), culminating in the same apical health outcome (effect) or effect syndrome (see Figure ES-2).
b) Similar (but not identical) toxic action: Different MIEs with a common shared downstream biochemical/biophysical process (i.e., a kinetic and/or dynamic KE), where the chemical "doses" add together (i.e., a dose-additive event); after the convergence, the shared KE(s) culminate(s) in a specific apical health effect or effect syndrome (see Figure ES-3 Chemicals D and E, for an example).
c) Same apical effect or effect syndrome: MIE and other key toxicokinetic and/or toxicodynamic processes unknown.
d) Same target organ.
e) Similar chemical structure: Implied action at the same MIE and implied similar toxicokinetics and/or toxicodynamics.
f) Similar DRC shape: Examples of curve similarity are when all components show evidence of a dose threshold or all show a linear (versus S-shaped) curve. Proportional toxicodynamics and toxicokinetics across the component chemicals is implied.

Within each type of evidence listed above, clearly there can be gradations.

Figure ES-2 and Figure ES-3 illustrate aspects of the evidence for similarity. Figure ES-2 depicts chemical clones exhibiting the same toxicokinetic properties, interacting with biological targets through the same MIE, eliciting the same sequence of KEs, and resulting in the same apical health outcome. The chemical concentrations themselves "add together" to elicit a response. Each of these chemicals could be placed in the same dose-additive group based on the evidence; confidence in this grouping will reflect the state of knowledge about the shared MOA.

Figure ES-3 depicts a hypothetical chemical mixture exhibiting more complex AOPs that show pathway convergence and pathway independence. While the three chemicals may not be intuitively dose-additive based on independence of action at the level of the MIE, knowledge of the common downstream process(es) suggests that it could be appropriate to group Chemicals D and E, as a consequence of exposure to a mixture of chemicals exhibiting convergent AOP. The pathway for Chemical F is seemingly toxicologically independent of the pathway for Chemicals D and E. Chemical F would generally not be included in the dose-additive group with Chemicals D and E; the joint toxicity of Chemicals D, E, and F could be estimated using an integrated addition approach.

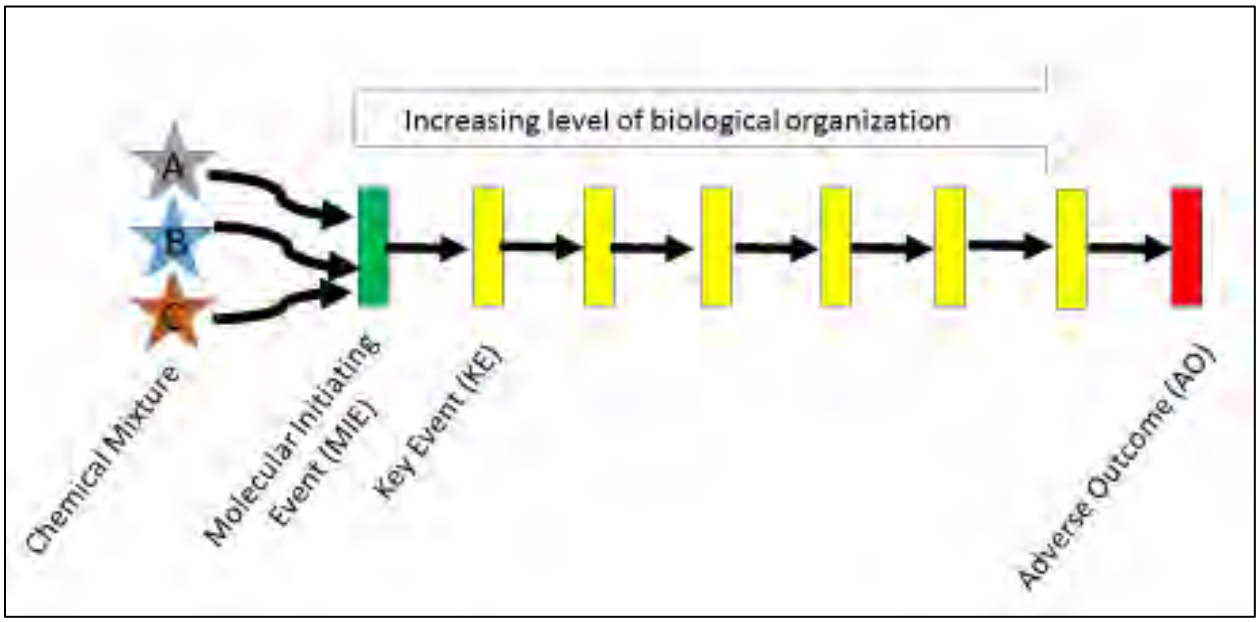


Figure ES-2. A common mode of action shared by a mixture of chemicals that act as toxicodynamic "clones" affecting an adverse outcome.

This diagram depicts a simple hypothetical MOA with one MIE (depicted as a green rectangle), which leads to a linear series of KEs (depicted as yellow rectangles). A mixture of three chemical stressors that act as toxicodynamic clones (symbolized as different colored stars; i.e., Chemicals A, B, and C) comprise a single dose-additive group that perturbs a biological function via a common molecular-level interaction (e.g., binding to a receptor). The paths of the chemicals to this initial toxicodynamic event are symbolized by different curved arrows to indicate potentially different toxicokinetic processes or differences in the way the chemicals perturb the MIE. Each chemical depicted has a specificity and affinity to the MIE. The initial perturbation can cause subsequent events to occur in sequence, where each KE represents increasing levels of biological organization, finally reaching the observed adverse outcome (AO). Each KE can be observed/measured and the progression toward the AO (depicted as a red rectangle) observed. The KE relationships (straight arrows in Figure ES-2) describe the conditions under which a particular biological change, represented as a KE, will trigger the next KE in the sequence.
AO = adverse outcome; KE = key event; MIE = molecular initiating event; MOA = mode of action.

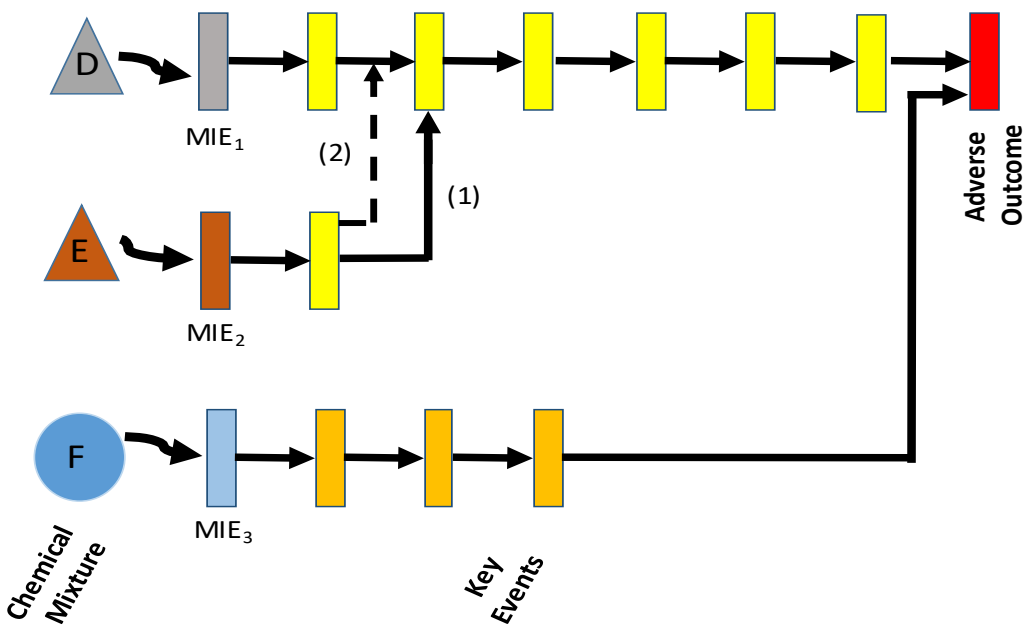


Figure ES-3. Diagram of hypothetical mode of action for a chemical mixture depicting pathway convergence (Chemicals D and E) and pathway independence (Chemical F).

Mixture Chemicals D, E, and F, represented by the gray and brown triangles and blue circle, respectively, induce the same AO via three different MIEs; the chemicals specifically perturb the corresponding MIE of the same color. The pathway of intermediate KEs and KERs (depicted using colored rectangles and black arrows, respectively) linking MIE_3 to the AO are distinct from the KEs associated with MIE_1 and MIE_2 and do not intersect until the AO. The convergence of the pathway for Chemical E to Chemical D may occur via one or more events/processes: (1) intermediate KE_1 for pathway E may be the same/similar toxicodynamic event as intermediate KE_2 in pathway D, resulting in amplification of that KE signal; and/or (2) intermediate KE_1 for pathway E may affect the toxicokinetics of constituents in pathway D such that KERs at one or more nodes along the pathway continuum are impacted, thus conferring signal modification (may be amplification or inhibition) and potentially impacting the overall AO response. The decision to include or exclude chemicals in a specific dose-additive group ultimately depends on where the strength and breadth of evidence supporting similarity of toxic action falls on a continuum and the level of evidence required in the assessment. (It is recommended that users of this document coordinate such decisions with the relevant U.S. EPA programs, offices, or regions.) Low evidence of toxic similarity (e.g., simply the same target organ or similar chemical structures) could potentially be adequate for screening assessments or applications of the HI, for example. Conversely, more detailed evidence of the same or similar toxicodynamics would be ideal for establishment of RPFs. Evidentiary bases between these two ends of the data-dependent continuum can be envisioned and whether such data suggest the chemicals comprise a single dose-additive group, multiple independent groups, or something more complicated, will require an assessment of the available toxicodynamic information and likely some scientific judgment.

AO = adverse outcome; HI = hazard index; KE = key event; KER = key event relationship; MIE = molecular initiating event; RPF = relative potency factor.

Section 3 concludes by illustrating the underlying toxicological pathway information for three different, component methods: dose addition, response addition, and integrated addition. Differences in the toxicodynamic information help select among these methods.

Section 4 focuses on component-based methods using an index chemical, but many concepts are discussed that also apply to other component-based approaches, such as response

surface models. Important to these discussions is the type and extent of evidence supporting toxicological similarity among the mixture component chemicals.

Some experimental data characteristics are often interpreted as supporting response addition methods, whereas dose-additive methods may actually be more appropriate in certain circumstances given recent toxicological information (e.g., increased understanding of AOP networks). Several concepts and examples are presented to highlight the types of evidence that would support the consideration of alternative versions of dose addition that do not require the dilution interpretation of toxicological similarity described in the *Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (U.S. EPA 2000c). For the least informative evidence of deviation from toxicological similarity, when the DRCs are not similar across components (as described above in the list of evidence for similar toxic action [see Item f) Similar DRC shape]), mixture response predictions from RPF models are subject to dependence on the choice of index chemical. In this case of dissimilar DRC shapes, several alternative dose-additive approaches, such as those based on Berenbaum's interaction index equation (e.g., Altenburger et al. 2000), have been shown to be especially useful in describing or predicting the mixture response (see **Section 4.1.3.1**). Berenbaum's equation is often used to test consistency with dose addition. For estimating the mixture response for environmental exposures near the tested exposure range, the transformed version of Berenbaum's equation known as the harmonic mean formula has seen wide success (see **Section 4.1.3.2**). Low-response modeling (see **Section 4.1.3.1**) may be a better alternative for low-dose extrapolation farther from the tested range, but it requires assumptions about the model's functional form and is hampered by the limited availability of low(er) dose-response data in the tested range. Finally, biologically based dose-response models offer the most accuracy and widest applicability to different exposure scenarios but also require detailed toxicokinetic and dynamic mechanistic knowledge about the components (see **Section 4.1.3.3**). With regard to the similarity of toxicological pathways, one conclusion is that dose addition, instead of response addition, can be applicable to chemicals for which MIEs and early or earlier KEs are independent, but for which pathways converge at a common KE prior to the apical health outcome (see **Section 4.1.4**).

Section 4.2 presents additional considerations for modeling the relative potency of continuous endpoints. Concerns are raised about addressing differences in component control groups by normalizing the measurements as fractions of the control means. For receptor-dependent signaling pathways, the effect of different high-dose maximal responses on mixture prediction, particularly for partial agonists, is discussed. Several approaches to this problem from the literature are also discussed and some significant limitations are noted. U.S. EPA has not endorsed any of these methods.

Section 4.3 identifies specific aspects of uncertainty and variability as they could be applied to the development or applications of the RPF approach. Recent advances in RPF concepts and calculation are more complicated and reflect many previously unstated uncertainties. **Section 4.3** underscores the consistency between the findings of this document regarding the importance of qualitative and quantitative uncertainty analyses and recommendations of previous documents published by U.S. EPA (notably U.S. EPA 2000c) and papers published in the open, peer-reviewed, scientific literature.

Section 5 describes opportunities for using NAMs to inform toxicokinetics and toxicodynamics when assessing health risks and hazards associated with environmental chemical mixtures. Since the 1990s, human health risk assessors have advanced the use of NAMs to inform chemical evaluation(s), as traditional animal toxicology approaches are time and resource-intensive. NAMs, including transcriptomics, proteomics, metabolomics, chemo and bioinformatics, cell-based bioactivity assays, reverse toxicokinetics, and nonmammalian alternative whole organism toxicity testing models, have been proposed for expediting human health risk assessment (e.g., see U.S. EPA 2020). The integration of NAM data to inform decisions regarding health outcome domain weight of evidence, MOA/AOP membership, and dose-response characteristics (e.g., dose additivity, response additivity, or deviations from additivity) could advance mixture risk assessment.

Almost every NAM approach proposed to date can provide qualitative evidence for membership in a dose-additive group. Based on the toxicological similarity (**Section 3**), some key questions for grouping chemicals that may be addressed by NAM assays include the following:

- Do chemicals "look similar" to one another (e.g., structurally or physicochemically);
- Do they share the same or similar profile of absorption, distribution, metabolism, and/or elimination (i.e., toxicokinetics);
- Do they share or induce the same or similar bioactivities; and ultimately,
- Do they result in or are they predicted to share a similar adverse health outcome?

Many chemical mixtures are now known to operate through complex biological networks (e.g., multiple different MIEs and pathways; as an example, see Figure ES-4). Addressing such complexity at this time is particularly difficult in a NAM data context because of the biological granularity at which hazard and dose-response could be characterized. Data may be available to evaluate whether the chemicals share an entire toxicodynamic pathway or share part of a toxicodynamic pathway (e.g., several shared KEs following the merging of pathways; see Figure ES-4). A key factor in forming dose-additive groups of chemicals is the dose and AOP process at which decisions regarding commonality are made.

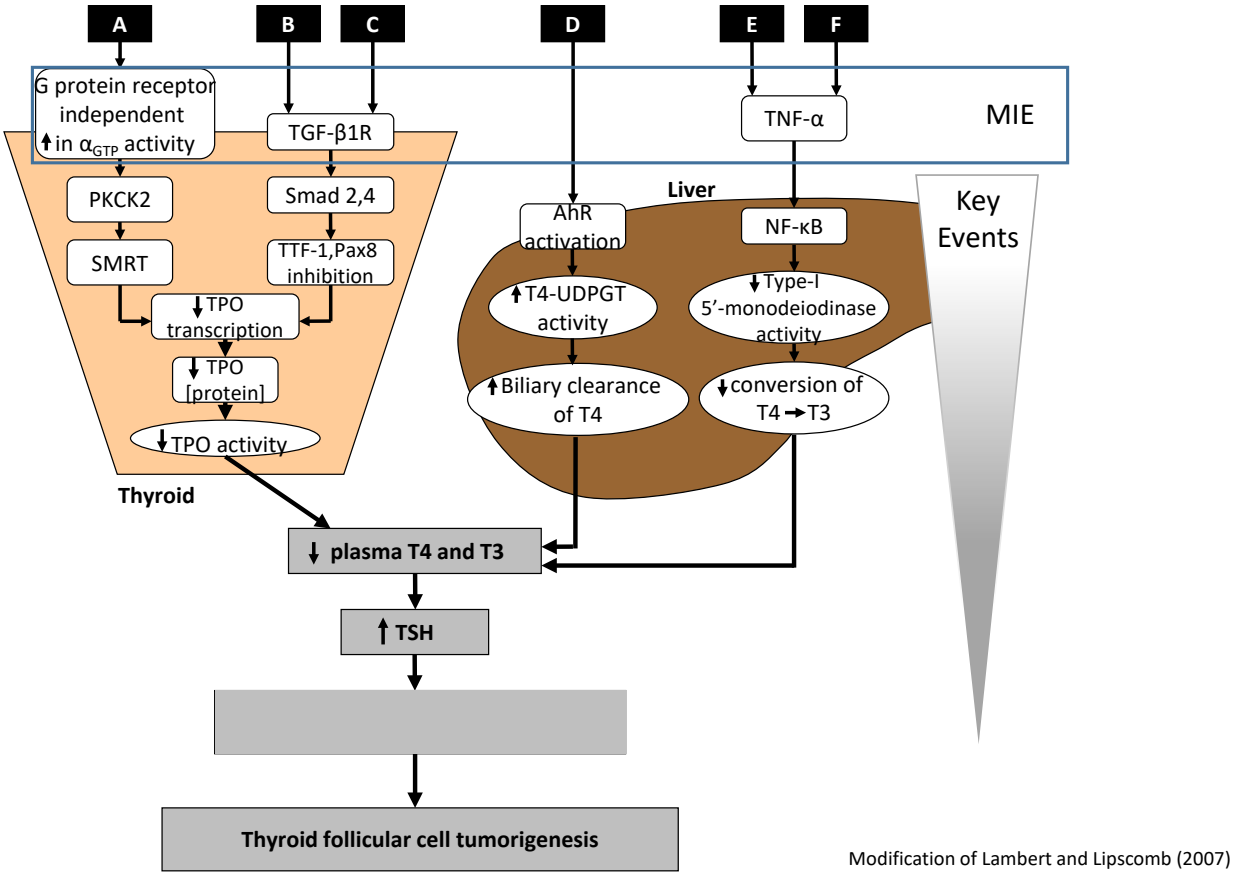


Figure ES-4. A hypothetical mixture of six chemicals induces thyroid follicular cell tumorigenesis via several related adverse outcome pathways.

While there is no apparent similarity across all chemicals at the MIE level, evaluation of potential KEs along AOPs reveals a convergence point/KE (e.g., decreased plasma levels of thyroid hormones T4 and T3) where all causal AOPs affect the same apical outcome through the same downstream KE.
AhR = aryl hydrocarbon receptor; AOP = adverse outcome pathway; GTP = guanosine-5′-triphosphate; KE = key event; MIE = molecular initiating event; NF-κB = nuclear factor kappa B; Pax8 = paired box 8; PKCK2 = protein kinase casein kinase 2; Smad 2,4 = mothers against decapentaplegic homolog 2 and 4; SMRT = silencing mediator of retinoic acid and thyroid hormone receptor; T3 = triiodothyronine; T4 = thyroxine; TGF-β1R = transforming growth factor beta-1 receptor; TNF-α = tumor necrosis factor alpha; TPO = thyroid peroxidase; TSH = thyroid stimulating hormone; TTF-1 = thyroid transcription factor 1; UDPGT = uridine 5′-diphospho-glucuronosyltransferase.

Once chemicals are qualitatively grouped together, determining whether and how the NAM-based concentration response data can be used to inform the hazard characterization or mixtures dose-response assessment is challenging. Interpreting how a given level of perturbation in a nonapical effect (e.g., an in vitro bioactivity concentration that is 50% of the maximum response) translates to the incidence or magnitude of adverse phenotypic health outcome(s) in a living organism can be uncertain.

NAM platforms offer the prospect of generating a tremendous "landscape" of structural, physicochemical, and molecular and cellular bioactivity information, particularly for chemicals

with little or no traditional hazard data available. However, as with apical outcomes, the limited availability of mechanistic or pathway-based mixtures studies hampers the evaluation of assumptions of additivity of the chemicals. The current reality is that NAM data that address toxicities will be generated primarily for individual chemicals, and component-based mixtures methods are key for predicting mixture toxicity[1]. However, as discussed in **Section 5.1**, application of non-apical NAM data in mixtures dose-response assessment may be challenging because of the difficulties identifying what constitutes a hazard.

The nexus between NAM data generation and assessment application has been significantly informed by the concept of Integrated Approaches to Testing and Assessment (IATAs) (OECD 2013; Tollefsen et al. 2014). An IATA facilitates the systematic integration of extant information with NAM data into evaluations of potential hazard(s) and concentration- or dose-response. IATAs also readily identify and provides an opportunity to fill data gaps. A key aspect of IATAs is the identification of critical pathways (i.e., AOPs/MOAs) associated with a given adverse health outcome (OECD 2017). The AOP project has made tremendous progress recently, but much work remains, particularly in identifying translational applications to risk assessment.

One such characterization approach might be AOP "footprinting." The overarching principle of AOP footprinting is the stepwise profiling and comparison of AOPs at the level of KEs moving backward from the most downstream KE to MIEs (Lambert 2022). This approach may help identify commonality among mixture chemicals at a level of biological organization that is more functionally relevant to a health outcome (e.g., significantly decreased androgen commonly leads to increased incidence of epididymal and testicular effects in male offspring).

Appendix A focuses on the numerical issues involved when applying the RPF method to a mixture. It provides additional explanation for some of the quantitative aspects of the RPF formula and discusses the alternative method that uses the Berenbaum equation. Examples are shown that use the U.S. EPA Benchmark Dose Software package.

The assessment of chemical mixtures continues to be an area of active scientific investigation. Several journal articles have appeared in the past 10 years involving new biological concepts and data as well as new approaches to statistical analysis of both classical and NAMs-generated data. One goal of this White Paper is to stimulate further advances in this multidisciplinary area of risk assessment of chemical mixtures.

[1]The diversity in mixture components and relative proportions of each across different environmental mixtures represents a significant challenge for human health risk assessment. This variability in mixtures leads to myriad potential exposures, making targeted toxicity testing of all variations of a given mixture impractical. However, due to greater flexibility in study design, resource efficiency, and timing, NAM approaches (e.g., high-throughput transcriptomics and cell bioactivity) provide an opportunity to evaluate a broader landscape of mixture exposures.

1. INTRODUCTION

Many human exposures involve concurrent or temporally sequential exposures to mixtures of chemicals; as such, the complexity of assessing joint toxicity[2] associated with multichemical exposures warrants continued advancement of methods and approaches. To address concerns over health risks from multichemical exposures, the U.S. Environmental Protection Agency (U.S. EPA) issued the *Guidelines for the Health Risk Assessment of Chemical Mixtures* in 1986 (U.S. EPA 1986), hereinafter termed the 1986 Chemical Mixtures Guidelines. These guidelines were followed in 2000 by the *Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (U.S. EPA 2000c), hereinafter termed the 2000 Supplementary Chemical Mixtures Guidance. These documents define a mixture as "any combination of two or more chemical substances, regardless of source or of spatial or temporal proximity, that can influence the risk of chemical toxicity in the target population" (U.S. EPA 1986; 2000c); this White Paper will use this definition.

The two earlier documents (U.S. EPA 1986; 2000c) also included descriptions of procedures for assessing health risks using information on whole mixtures and on the integration of hazard and dose-response information across component chemicals of a mixture. In this White Paper, U.S. EPA shares and discusses its extensive experience and that of other organizations and investigators in implementing component-based mixture assessment methods that utilize dose addition. Dose addition provides a simple mathematical approach that attempts to estimate joint toxicity associated with complex interactions among biological systems and combinations of chemicals from exposures in the environment. Mixture assessment methods based on dose addition have been used to assess both cancer risks and noncancer hazards when based on an equivalent exposure to an index chemical.

The development of the current document is consistent with U.S. EPA's interest in advancing chemical mixture risk assessment, as part of an overarching effort to advance the broader area of cumulative risk assessment that includes chemical and non-chemical stressors. Accordingly, it is intended to address the following: (1) the Agency's experience applying dose-additive methods in human health risk assessment of environmental chemical mixtures; (2) publications of methods based on dose addition by U.S. EPA's Office of Research and Development and U.S. EPA's programs, offices, and regions; (3) publications in the scientific literature since 2000 that are related to dose addition; and (4) advances in the fields of

[2]Joint toxicity refers to the biological response associated with exposure to a mixture of chemicals in combination; the term inherently includes the toxicokinetics and toxicodynamics of the component chemicals and is a key determinant for assumptions regarding dose-response evaluation of the activity of chemicals in a component-based method (e.g., dose additivity, synergism, and antagonism).

experimental design, biomathematics, high-throughput (HTP) assays and computational toxicology, molecular and cellular biology, and biochemistry that have improved the scientific understanding of kinetic and dynamic responses to chemicals at subcellular, cellular, tissue, and organ levels. As a White Paper, this document is not intended to provide specific guidance.

Over two decades ago, the toxicology and human health risk assessment communities recognized the need for advancing the use of new approach methodologies (NAMs) to inform chemical evaluation(s), as traditional toxicology approaches were and are time and resource-intensive. U.S. EPA and the European Chemicals Agency (ECHA) define NAMs as any technology, methodology, approach, or combination that can provide information on chemical hazard and risk assessment without the use of animals, including in silico, in chemico, and in vitro approaches (ECHA 2016; U.S. EPA 2018b). Further, in the report "Toxicity Testing in the 21st Century: A Vision and A Strategy," the National Academy of Sciences (NAS) recognized that testing methodologies to inform the understanding of biological activities of environmental chemicals are evolving from "a system based on whole-animal testing to one founded primarily on in vitro methods that evaluate changes in biologic processes using cells, cell lines, or cellular components, preferably of human origin" (NRC 2007). Recognizing the current mixture assessment concepts and methods described in Sections 1 to 4 of this document are evolving, the Technical Panel developed Section 5 to describe opportunities for some potential uses of NAMs to inform the toxicokinetics and toxicodynamics of mixtures of environmental chemicals.

Finally, this White Paper also addresses uncertainty and variability, primarily as these concepts pertain to the development or applications of mathematical models based on dose addition. U.S. EPA (2001c) described variability as "true heterogeneity or diversity that characterizes" a response in a population. Further study (e.g., increasing sample size) will not reduce variability, but it can provide greater confidence (e.g., lower uncertainty) in quantitative characterizations of variability." U.S. EPA (2001c) described uncertainty as a "lack of knowledge about specific variables, parameters, models, or other factors... Uncertainty may be reduced through further study." In some cases when the term "uncertainty" is used, it can refer to both uncertainty as described here and variability (specifically, see Section 4.3).

1.1. DOCUMENT PURPOSE

This document describes the Agency's use of methods based on dose addition when conducting risk assessments involving multiple chemicals and discusses specific issues associated with these methods given the Agency's experience and scientific publications since the publication of the 2000 Supplementary Chemical Mixtures Guidance. This document includes presentation, clarification, and discussion of the following:

- Current practices and methods within U.S. EPA that are based on dose addition and which address health risks and health hazards posed by exposure to mixtures of chemicals (see Section 2).
- Considerations for grouping chemicals when applying methods based on dose addition (see Section 3).
- Issues affecting the prediction of chemical mixture risks with dose-additive methods (see Section 4).
- Potential integration of data generated by NAMs into chemical mixture risk assessment methods based on dose addition (see Section 5).
- Methods for evaluating constant relative potency[3] from component dose-response modeling output (see Appendix A, Sections A.1 and A.2) and for predicting response to the mixture when the shapes of the dose-response models differ (see Section A.3).

The potential uses of the following data sources in dose-addition-based mixture methods are of interest: HTP platforms such as toxicogenomics (e.g., transcriptomics, the characterization of gene expression changes in a cell or tissue following exposure to a chemical or chemical mixture), chemo- and bioinformatics (e.g., structure-activity relationships, read-across, data-mining techniques), and cell-based bioactivity screening assays (e.g., ToxCast, Tox21). As most NAM development was in its infancy in 2000, these topics were not addressed in the U.S. EPA Supplementary Chemical Mixtures Guidance.

This document's intended audience includes U.S. EPA human health experts/risk assessors as well as consultants and contractors who participate in Agency evaluations of health risks posed by chemical mixtures, particularly those involving dose-additive methods. Risk assessors, including those from state agencies, academia, and industry, who develop such chemical mixture risk assessments in accordance with U.S. EPA policies and procedures, may find the information in this document useful. In addition, toxicologists who plan to conduct toxicokinetic and/or toxicodynamic studies on mixtures that are meaningful for evaluation of hazards or risks or those who plan to evaluate assumptions inherent in the methods described herein may find this document useful. Risk managers and decision makers in the Agency might benefit from reading this document because it explains terminology, describes dose-additive methods, and discusses issues encountered when applying these methods. This document is not intended as a basic reference for readers new to the assessment of health risks following exposures to mixtures of chemicals in the environment; for the overview and background of such

[3]Constant relative potency indicates that the potency of one chemical relative to that of another is unchanged over a specified range of doses.

procedures, readers are referred to previous publications (MacDonell et al. 2018; Schaaper et al. 2020; Torres and Bobst 2015; U.S. EPA 2000c).

Unless otherwise noted, the mathematical models described in this document are considered generally applicable to assessments involving exposed human populations including individuals at any life stage. While the models may be applicable across life stages, the analyst will need to evaluate the relevance of the available toxicological or epidemiological data to a particular life stage, with specific consideration of biological changes (e.g., developmental changes and aging-related changes) during relevant time windows of exposure for the chemical mixture. U.S. EPA recommends that analysts coordinate such decisions with the appropriate U.S. EPA programs, offices, and regions as well as in consultation with existing U.S. EPA guidance/guidelines.

This document does not address directly the analysis of human epidemiological data and associated statistical models that can be used to evaluate such data. Further, this White Paper does not address issues related to "complex" or "whole" mixtures, as it focuses on component chemical-based methods that rely on an assumption of dose addition. Similarly, this report does not consider the comparison of responses predicted based on dose-additive models to observed responses to whole mixtures (i.e., sometimes referred to as validation experiments), for which several other reports can be consulted (e.g., ATSDR 2023). The document also is not intended to serve as a comprehensive repository for previous U.S. EPA mixture assessments that relied on assumptions of dose addition.

As emerging topics mature, U.S. EPA may update or supplement this document or develop application-specific guidance focused on a particular method. U.S. EPA recommends that users of this document coordinate with the relevant U.S. EPA programs, offices, and regions for further information regarding the application of the methods described herein.

1.2. U.S. EPA'S REGULATORY PURVIEW AND OVERVIEW OF CHEMICAL MIXTURES RISK ASSESSMENT APPROACH

Several laws direct U.S. EPA to address health risks posed by exposures to chemical mixtures, including the Toxic Substances Control Act (TSCA) of 1976 (amended 2016); the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) of 1980; the Superfund Amendments and Reauthorization Act (SARA) of 1986 and amendments in 2002 (CERCLA 2002; SARA 2002), commonly referred to as Superfund; the Clean Air Act Amendments of 1990 (CAA 1990); the Safe Drinking Water Act Amendments of 1996 (SDWA 1996); and the Food Quality Protection Act (FQPA) of 1996 (FQPA 1996). Both the 1986 Chemical Mixtures Guidelines (U.S. EPA 1986) and the 2000 Supplementary Chemical

Mixtures Guidance (U.S. EPA 2000c) were developed, in part, to be responsive to these laws.[4] In developing information for exposures to chemical mixtures, risk assessors and risk managers in U.S. EPA's programs, offices, and regions currently implement environmental laws through regulations that rely on the guidance and methods articulated in the 1986 Chemical Mixtures Guidelines and the 2000 Supplementary Chemical Mixtures Guidance. The science-based methods, described in these two U.S. EPA documents and discussed in Section 2 are essential to evaluate human health risks and hazards from exposures to mixtures of chemicals in the environment.

Text Box 1-1 briefly summarizes these two U.S. EPA chemical mixtures guideline and guidance documents, and Text Box 1-2 provides an overview of some U.S. EPA program-specific guidance regarding dose addition.

Text Box 1-1

U.S. EPA Guidelines and Guidance Pertaining to Models Based on Dose Addition

(2000c) Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures*. This guidance provides procedures for analyzing data, including using whole-mixture data on a toxicologically similar mixture, generalized procedures for mixtures involving classes of similar chemicals, and methods for defined mixtures, including incorporating information on toxicologic interactions to modify a hazard index.*

(1986) Guidelines for the Health Risk Assessment of Chemical Mixtures. *The purpose of this document is to generate a consistent approach for evaluating data on the effects of chemical mixtures, emphasizing the underlying scientific principles necessary for assessing health risk from chemical mixture exposure.*

Both the 1986 Chemical Mixtures Guidelines and the 2000 Supplementary Chemical Mixtures Guidance (U.S. EPA 1986; 2000c) recommend three data-dependent approaches to quantify health risks and health hazards of a chemical mixture.[5] Figure 1-1 highlights the three data-dependent approaches used to estimate the health risks and hazards associated with a mixture of concern (target mixture). Figure 1-1 depicts broad consideration of

[4]In 1983, the NAS NRC published *Risk Assessment in the Federal Government: Managing the Process* (NRC 1983). In that book, NAS recommended that federal regulatory agencies establish "inference guidelines" to ensure consistency and technical quality in risk assessments and to ensure that the risk assessment process was maintained as a scientific effort separate from risk management. The U.S. EPA (1986) and U.S. EPA (2000c) mixture risk documents are responsive to that recommendation.

[5]Improved information usually results when all three types of data are available, used, and synthesized, with the attendant uncertainties summarized. For example, differences between whole-mixture-based assessments and component-based assessments could identify exposure ranges in which significant departure from the additivity-based risk estimate are expected.

all relevant data sources: mixture of concern, similar mixture[6], and components. There is no rigid, single, hierarchical decision approach offered herein. Following information collection, all of the data from the different approaches, including the component and interaction data, are evaluated and considered together. This expanded evaluation, in particular, facilitates generalization to other scenarios. Due to the lack of whole mixture data, component-based methods are used more frequently, and many of these methods are based on dose addition. The remainder of this document focuses on the use of component-based, dose-additive methods for the evaluation of mixtures of chemicals.

Figure 1-1. Schematic for estimating risk associated with a mixture of concern.

This figure depicts the broad consideration of all relevant data sources: mixture of concern, similar mixture, and components. All of the data from the different sources are evaluated and considered together.

[6]While U.S. EPA (2000c) describes general principles for judging sufficient similarity, the document does not address specific methods. Since its publication, a number of journal articles have reported methods that address whether mixtures are toxicologically similar (Catlin et al. 2018; Feder et al. 2009a; Feder et al. 2009b; Marshall et al. 2013; Ryan et al. 2019).

Text Box 1-2

Examples of Applications of Agency-Wide Chemical Mixture Guidance and Related Guidance Developed by U.S. EPA Offices and Programs

(2016) Pesticide Cumulative Risk Assessment Framework for Screening-Level Analysis. *This document describes a tiered approach for cumulative screening-level residential exposure analysis, including considerations of available information concerning the cumulative effects of pesticides and other substances that have a common mechanism of toxicity.*

(2015b) Technical Support Document: EPA's 2011 National-Scale Air Toxics Assessment. *The hazard index (HI) and simple risk addition are recommended for noncancer and cancer effects, respectively.*

(2010) Recommended Toxicity Equivalence Factors (TEFs) for Human Health Risk Assessments of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and Dioxin-Like Compounds. *This document recommends that the TEF methodology, a component-mixture method, be used to evaluate human health risks posed by these mixtures, using TCDD as the index chemical.*

(2006c) Exposures and Internal Doses of Trihalomethanes in Humans: Multiroute Contributions from Drinking Water. *The document includes simulation predictions (for absorbed dose analyzed for each chemical as a function of route (dermal, ingestion, and inhalation). For each chemical, a table containing the absorbed dose is presented as a function of route, population group, and percentile of the population. In addition, the cumulative distribution function is plotted along with histograms.*

(2006b) Considerations for Developing Alternative Health Assessment Approaches for Addressing Multiple Chemicals, Exposures, and Effects. *Examples of biostatistical modeling to combine dose-response information, partitioning mixtures into common modes-of-action subclasses for specific exposure scenarios or to develop toxicity values. The document includes three examples.*

(2005b) Human Health Risk Assessment Protocol for Hazardous Waste Combustion Facilities, Final. *The document discusses the HI and the multipathway HI.*

(2003b) The Feasibility of Performing Cumulative Risk Assessments for Mixtures of Disinfection By-Products in Drinking Water. *This document evaluates the feasibility of conducting a cumulative risk assessment for drinking water disinfection by-product mixtures by combining exposure modeling results with the cumulative relative potency factor (RPF) risk assessment approach.*

(2003a) Developing Relative Potency Factors for Pesticide Mixtures: Biostatistical Analyses of Joint Dose-Response. *Development of the biological concepts and statistical procedures for improving applications of the RPF approach for the Office of Water's Contaminant Candidate List and under the Food Quality Protection Act.*

Text Box 1-2 (Continued)

Examples of Applications of Agency-Wide Chemical Mixture Guidance and Related Guidance Developed by U.S. EPA Offices and Programs

(2002a) Guidance on Cumulative Risk Assessment of Pesticide Chemicals That Have a Common Mechanism of Toxicity. *This document provides guidance on assessing cumulative risks for pesticide chemicals that act by a common mechanism of toxicity.*

(2001c) Risk Assessment Guidance for Superfund (RAGS), Volume III, Part A—Process for Conducting Probabilistic Risk Assessment. *This document provides guidance on applying probabilistic analysis to risk assessments to support remedial action decisions at Superfund hazardous waste sites.*

(1993) Provisional guidance for quantitative risk assessment of polycyclic aromatic hydrocarbons. *This provisional document presents an approach (based on dose addition) for assessing cancer risks for selected polycyclic aromatic hydrocarbons that* U.S. EPA *had characterized as probable human carcinogens.*

Risk Assessment Guidance for Superfund, Volume I. Human Health Evaluation Manual. *This collection of documents provides guidance on risk assessment for Superfund sites and includes a discussion of the HI approach along with additional guidance that addresses dose addition:*

> ***(1989b) Part A—****Chapter 8 describes procedures for calculating aggregate hazards from multiple chemicals and pathways.*
>
> ***(1991a) Part B—****This document discusses calculation of preliminary remediation goals considering cancer and noncancer health effects.*
>
> ***(1991b) Part C—****This document provides guidance on human health risk evaluations for remedial alternatives that are conducted during the feasibility study, during remedy selection and documentation, and during and after remedy implementation.*
>
> ***(2001b) Part D—****This document provides guidance on risk assessment planning, reporting, and review throughout the Comprehensive Environmental Response, Compensation, and Liability Act remedial process, from scoping through remedy selection and completion and periodic review of the remedial action. The tables include formats for presenting calculations of noncancer HI and organ-specific HI.*
>
> ***(2004) Part E—****This document provides a consistent methodology for assessing the dermal pathway for Superfund human health risk assessments. The document describes calculation of noncancer HIs for dermal exposures.*
>
> ***(2009b) Part F—****The document recommends an approach for developing information necessary for risk assessment and risk management decision making at waste sites involving potential risks and hazards from inhalation exposures.*

2. RISK ASSESSMENT OF CHEMICAL MIXTURES USING DOSE ADDITION

The process of human health risk assessment of chemical mixtures is similar to that of single chemicals. The main difference is that risk assessment for chemical mixtures includes the consideration of joint toxic action (i.e., how to describe and/or quantify the consequences from the joint chemical exposure in ways that reflect the interplay of the toxicological similarities and differences among the component chemicals in the mixture). To enhance the transparency and precision of the concepts and formulas used by U.S. EPA, certain terms in this report are given definitions that in some cases are more restrictive or carry different assumptions than corresponding definitions in use outside of U.S. EPA. In this report, a mixture "risk assessment" is the process of evaluating the available hazard and dose-response information to estimate health consequences from exposure to a chemical mixture; these health consequences can be characterized in terms of risk or in terms of comparisons of exposure to a health-based benchmark (sometimes referred to as a health hazard evaluation). Consistent with past Agency guidance and current practice (e.g., U.S. EPA 2023b), the term "risk" is defined here as the probability of health consequences, particularly adverse effects, occurring in an exposed individual or population, recognizing that risk may be presented only qualitatively. The term "adverse" is defined as in the Integrated Risk Information System (IRIS) glossary (U.S. EPA 2023b): "a biochemical change, functional impairment, or pathologic lesion that affects the performance of the whole organism, or reduces an organism's ability to respond to an additional environmental challenge." The term "hazard" is defined here to represent the nature of the adverse health effect estimated for the exposed population, including some characterization of the expected severity (as a function of exposure dose and/or duration and/or route), which also is consistent with past Agency guidance and practice (e.g., U.S. EPA 2000c). The term "toxicological interaction" is defined here as the deviation from the mixture risk or hazard predicted using the concept of dose addition (e.g., synergy; antagonism). In the following sections, formulas and examples are presented to expand on and explain these terms and their applications in U.S. EPA mixture risk assessment; some of these estimate hazard and others calculate risks (i.e., integration of hazard and exposure). Both types of results will be clarified throughout this document.

2.1. ADDITIVITY CONCEPTS AND RELATED DEFINITIONS USED BY U.S. EPA

Additivity-based methods are used to estimate the probability of toxic effect or extent of the health hazard (e.g., severity of noncancer health effects such as deleterious changes in continuous measures) associated with exposures to mixtures using data on the individual mixture components. In previous guidance on component-based mixture approaches, U.S. EPA relied on

two main concepts: simple similar action and simple independent action, as described by Bliss (1939) and Finney (1971), respectively.[7] U.S. EPA has applied these two concepts using formulas for dose addition and for response addition.

• *Simple similar action applies to chemicals that are toxicologically similar and cause a common health effect.* Simple similar action means that chemicals act as if they are dilutions or concentrations of each other, eliciting the common effect by the same mechanism of action (U.S. EPA 2000c). Methods based on dose addition generally are applied when assuming that chemicals act through simple similar action.

• *Simple independent action applies to toxicologically dissimilar chemicals that cause a common health effect, where "dissimilar" is defined as the chemicals having different toxic mechanisms.* This means that the responses to different chemicals are independent events (U.S. EPA 2000c). Response addition is generally applied when it is established that chemicals are toxicologically dissimilar.

The component-based methods U.S. EPA has used for additivity of dose, response, or both are shown in Figure 2-1. The different methods involve different aspects of joint toxicity (the effects from joint exposure to multiple chemicals). Furthermore, many joint exposures involve chemicals that are not completely similar (nor completely independent) across all endpoints, but show characteristics consistent with the concepts of both dose addition and response addition, and might be better addressed with integrated addition (see Section 2.2.4). Improved understanding of the mixture risk also might be obtained by considering all the methods and their assumptions (U.S. EPA 2000c). Alternative approaches also could be considered that involve complex concepts of additivity (see Sections 4 and 5) or biologically based models that explicitly include mechanisms of interaction (see Section 4.1.3.3).

[7]Other organizations, including the United Kingdom Committee on Toxicology (COT 2002), the European Food Safety Authority (EFSA 2013), and the World Health Organization (WHO)/International Program on Chemical Safety (WHO/IPCS 2009), have defined these terms similarly.

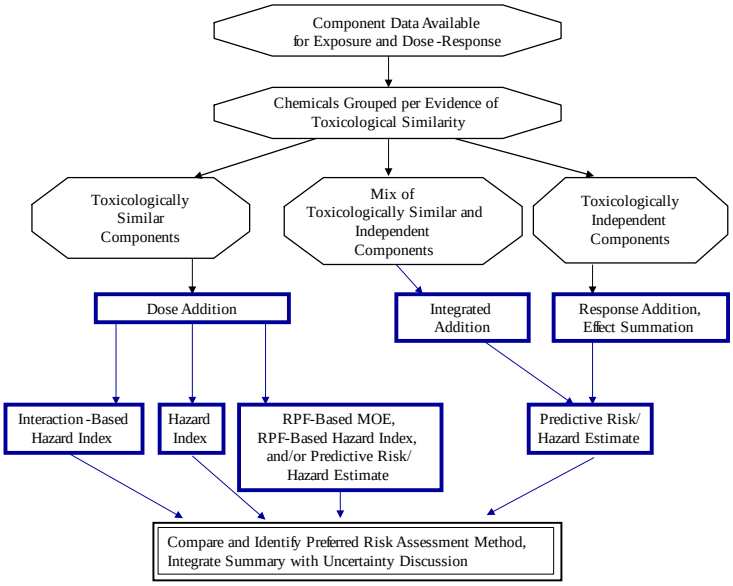


Figure 2-1. Flow chart for evaluating chemical mixtures using additive methods.

Path selected is based on the strength of the relevant evidence for toxicological similarity and toxicological independence. Rectangles indicate specific component-based assessment methods; octagons indicate results of data gathering or evaluation. Methods are described in subsequent subsections of Section 2.
MOE = margin of exposure; RPF = relative potency factor.

2.1.1. Similarity of Toxic Action

Dose addition and response addition are U.S. EPA's most commonly used component-based approaches applied to mixtures of chemicals exhibiting simple similar action and simple independent action, respectively (U.S. EPA 2000c). Further, U.S. EPA has stated that "the primary criterion for choosing between dose addition and response addition is toxicological similarity among the chemicals in the mixture," noting that this choice "should be based on information about the toxicological and physiological processes involved, the single-chemical dose-response relationships, and the type of response data available" (U.S. EPA 2000c). The same guidance document also explains that the concept of similarity "represents a general knowledge about the action of a chemical or a mixture and can be expressed in broad terms such as at the target organ level in the body (e.g., enzyme changes in the liver)." In the current document, consistent with U.S. EPA (2000c) guidance, the concept of *toxicological similarity* is used as an overarching term with a wide range of specificity, which allows similarity judgments to be tailored to both the specific goals of the risk assessment and the availability of information across mixture components. The concept of *similarity of toxic action* is more focused, usually relating to the key events (KEs) or toxic mechanisms, and thus is consistent with the concept of

simple similar action. An important step is then the judgment that the evidence for similarity is strong enough to support the use of specific dose-additivity methods. For example, the hazard index (HI) can be applied with minimal evidence of similarity, such as a common target organ. The relative potency factor (RPF) methods can usually be applied only when more extensive evidence of similarity exists (e.g., common adverse outcome pathway [AOP], mode, or mechanism of action). Decisions regarding when it is more appropriate to use one dose-additive method versus another are specific to the program office conducting the assessment and depend on both regulatory requirements and the purpose(s) of the assessment.[8]

The information used to inform similarity could vary widely in availability and extent across the chemicals comprising a mixture. Details pertaining to structure, physicochemistry, environmental fate and transport, and toxicity (includes kinetics and dynamics) are all useful data elements for informing grouping of chemicals; however, similarity in toxic action is key among these elements. Data that could potentially inform similarity in toxic action may be collected at different levels of biological organization (e.g., subcellular, tissue, organ), through different types of studies (e.g., in vitro or in vivo), across different test species, and/or for human populations; or, similarity among mixture chemicals could be inferred based upon shared structure, physicochemical properties, and/or absorption, distribution, metabolism, and elimination characteristics (ADME; also referred to as toxicokinetics). Collectively, these alternative toxicity testing assays, platforms, and data are referred to as new approach methodologies (NAMs). Several articles on similarity in toxic action focus on toxicodynamics, using conceptual terms such as mode of action (MOA), mechanism of action, toxicity pathway (TP) and more recently, AOPs (U.S. EPA 2017). Included in those articles are descriptions of toxicodynamic processes following exposure to chemicals from the environment (Altenburger et al. 2004; Hertzberg et al. 2013; Johns et al. 2012; Mwanza et al. 2012; SCHER 2012; Tan et al. 2011). MOA, TP, and AOP are similar in that the basic objective across these constructs is to collect and assemble toxicodynamic data in a source to health outcome continuum, from the molecular initiating event (MIE), through one or more intermediate KE(s), culminating in a health outcome. However, there are important distinctions, particularly between MOA/TP and AOP, primarily in that MOA/TP entails more descriptive detail and systematic evaluation (e.g., Bradford Hill criteria) on a chemical-by-chemical-specific basis versus the more wholistic non-chemical-specific assembly of toxicodynamic evidence into AOPs. These toxicodynamic concepts and several examples are further discussed in Sections 3, 4, and 5. Characteristics useful for deciding similarity of toxic action are addressed in Section 3.2.

[8]For example, U.S. EPA (2002b) describes the development of RPFs for organophosphate pesticides.

2.1.2. Types of Additivity in Mixture Risk Assessment

The application of additivity in chemical mixture risk assessment involves the dose-response assessment or the risk characterization steps. Thus, the methods involve adding some measure of dose or response. The methods vary in the assumptions that motivate the dose-response formulas used (Hertzberg and Mumtaz 2018). The component-based approach actually used should be selected based on the quality of the available data and reference values. Such data-driven decisions should point to one of the three additivity approaches as most appropriate (dose addition, response addition, integrated addition). Within an approach category, there are different formulas and methods available. For dose addition, the available formulas include the HI and its variants, the RPF formula and its TEF variant, and the general multivariate dose-additive response surface model. Data availability, data quality and the purpose of the assessment should dictate which specific approach to use, with the final choice made in coordination with the appropriate U.S. EPA program office. This section includes the following five subsections: Dose Additivity (Section 2.1.3), Response Additivity (Section 2.1.4), Effect Summation (Section 2.1.5), Interaction as Deviation from Additivity (Section 2.1.6), and Integrated Addition (Section 2.2.4).

2.1.3. Dose Additivity (Finney Definition)

Dose addition is the approach used by U.S. EPA to predict the response to a mixture under conditions of simple similar action. Historically, this has been interpreted by thinking of the mixture as a single chemical solution comprised of various dilutions of the same chemical (Bliss 1939), an interpretation some call the Finney definition because of the author's early publications that included a formula for mixture response based on equivalent doses and constant relative toxic potency between mixture constituent chemicals (Finney 1942). A more general definition of dose addition that does not assume constant relative potency was later promoted in several articles by Berenbaum (1977; 1985; 1989). Methods based on Berenbaum's equation (Berenbaum 1985) are described in Section 4. Mixture risk methods used by U.S. EPA have followed the Finney definition; applications are easier and the dilution concept is easy to explain compared with Berenbaum's definition, which has no simple biological interpretation. U.S. EPA (2000c) explains further that dose addition applies "when the components act on similar biological systems and elicit a common response." When both conditions occur (similar biological system and elicitation of a common response) and no evidence is found to the contrary, this scenario can be viewed as being consistent with simple similar action.

The dilution definition implies that all components of the mixture would have similarly shaped dose-response curves (DRCs), which means that once each component dose (on the *x*-axis) has been scaled for toxic potency, the component response curves will be identical in

shape, that is, geometrically congruent (Hertzberg et al. 2013). When plotted using log(dose) the component curves are parallel, having a constant horizontal distance between them. In the case of such congruency, the prediction model for the response to the mixture would follow the same dose-response model as the components. For example, consider a binary mixture using a simple three-parameter exponential model for each component. For Chemical 1, with y = response and d = dose, the model is:

$$y_1(d_1) = \gamma + \exp(\alpha + b_1 d_1) \tag{2-1}$$

where the definitions of the parameters γ, α, and b are not important to this example of the method, except b_1 is related to the toxicological potency of the first chemical. Because of the common model property, Chemical 2 would have the same dose-response function except $b_1 d_1$ would be replaced by $b_2 d_2$, where the different dose coefficients (b_1 versus B_2) reflect differences in toxicological potency. The estimated response for the mixture with component doses (d_1, d_2) is then the same model as in Eq 2-1 but with the component dose term (e.g., $b_1 d_1$) replaced by a linear combination of the component doses ($\beta_1 d_1 + \beta_2 d_2$). This prediction model (Eq 2-2) is the classical dose addition formula.

$$y_{MIX}(d_1, d_2) = \gamma + \exp(\alpha + \beta_1 d_1 + \beta_2 d_2) \tag{2-2}$$

Note that Eq 2-2 is the same mathematical function as Eq 2-1 except for the linear combination of the two doses instead of the single dose term (i.e., the other two parameters [γ, α] are the same in both equations). Greek letters (β_i) are used for the dose coefficients in the mixture model to distinguish them from their counterparts (b_i) in the single chemical models. In practice, the model in Eq 2-1 would be estimated from data on Chemical 1 alone while that of Eq 2-2 would be estimated from data on the binary mixture (where the component doses are known). Ideally, the two dose coefficients for the same chemical would be statistically indistinguishable (e.g., the hypothesis that $b_1 = \beta_1$ is not rejected).

An important property of models based on dose addition is that they can predict effects of the mixture even when all the individual component chemical exposures are subthreshold (e.g., below their individual no-observed-adverse-effect levels [NOAELs]). In these models, the sum of the scaled component doses can exceed the equivalent threshold dose of the mixture and result in a detectable response, which has been supported experimentally (Jonker et al. 1996;

Silva et al. 2002). Note, however, that this observation is suggestive but not diagnostic of dose addition.[9]

The additivity concept for mixtures of toxicologically similar components is also applied using concentration addition. The mathematics of the exposure-response relationships are identical to those for dose addition, regardless of whether the Finney or Berenbaum definition is used. The distinction is that exposure is a concentration, usually either in the exposure media (e.g., ambient air) or in the target tissue.

2.1.4. Response Additivity

Under simple independent action, the mixture chemicals cause the same specific effect (e.g., hepatocellular carcinoma) or general effect (e.g., liver toxicity), and some measure of the toxic impact of each chemical is summed, assuming each impact is an independent response. That is, simple independent action implies that the presence of one chemical in the body has no influence on the toxic action of other chemicals in the body. There are two types of mixture risk assessment methods based on simple independent action: response addition and effect summation. The most commonly applied method is response addition (sometimes termed independent action in the toxicology literature), which uses toxicity measured as the probability (risk) of, or population fraction showing the health effect of concern. In the response addition method, the response to the mixture is predicted by summing the risk estimates for the mixture components under the law of statistical independence.[10] Using r_i for the i^{th} component risk, the formula for predicting the *n*-chemical response to the mixture probability (r_{mix}) for simple independent action is then:

$$r_{mix}(d_1,\ldots,d_n) = 1 - \prod_{i=1}^{n}\left(1 - r_i(d_i)\right) \tag{2-3}$$

The formula for a binary mixture is:

$$\begin{aligned} r_{mix}(d_1, d_2) &= 1 - \left(1 - r_1(d_1)\right)\left(1 - r_2(d_2)\right) \\ &= r_1(d_1) + r_2(d_2) - r_1(d_1)r_2(d_2) \end{aligned} \tag{2-4}$$

[9]Undetected responses for individual chemicals (because of low study power) affecting the same or a similar endpoint and operating through an independent MOA can become detectable when the responses add up for a mixture of those chemicals.

[10]Zero correlation of individual susceptibilities for the independent TPs is an assumption when using response addition. If there is evidence contrary to this assumption, an adjustment for partial correlation has been suggested (Bliss 1939; Hewlett and Plackett 1959).

At low risk levels (e.g., $r_i < 0.01$), the mixture risk is roughly the simple sum of the component risks (e.g., $r_1 + r_2$ for a binary mixture).[11]

Note that in contrast to the dilution principle of dose addition used by U.S. EPA, with response addition the component dose-response models are not explicitly shown because only the response at each component dose level being assessed is used. Thus, there is no constraint on the shape or slope of the dose-response function for each component. For example, one function may be linear with no threshold dose and another exponential with a threshold dose.

2.1.5. Effect Summation

Effect summation is another independence-based mixture assessment method. It involves adding the actual biological measurements associated with the individual mixture component doses (e.g., sum the incremental changes in diastolic blood pressure caused by each of the components). Effect summation is rarely employed in human health risk assessment but could be useful for small changes caused by each chemical, in part because the interpretation is clear. Effect summation methods are constrained to small incremental changes to preclude biologically implausible effect levels and thus are most appropriately used when the expected toxicity measurements are low (U.S. EPA 1986; 2000c).

In a different approach for measured effects, some investigators have scaled the toxicity measurement to a [0,1] range and then applied Eq 2-3 to those scaled measures, which appears to work fairly well in some circumstances (Backhaus et al. 2000; Backhaus et al. 2004). When this data transformation is used, however, the term, $1-r_i(d_i)$ in Eq 2-3 loses its biological meaning as the fraction of the population unaffected by the first chemical. Applying Eq 2-3 to such transformed responses is then interpreted as the use of a simple empirical model that is not linked to toxicological independence nor to the summing of component effects.

2.1.6. Interaction as Deviation from Additivity

The U.S. EPA (2000c) mixture guidance has defined toxicological interaction for risk assessment purposes as departures from either dose addition or response addition, with the most common interaction types being synergism (i.e., greater response than expected) or antagonism (i.e., less response than anticipated). Such a definition allows direct comparison of the additive risk estimation with one that incorporates interactions, including measurements of the response to the whole mixture. Several examples of toxicological interaction have been published, but variations in definitions and in the extent of quantification have limited their use in component-based health risk assessment of mixtures. Some of these examples are described in

[11]Under independence, whether using response addition or effect summation, when the components are all at subthreshold doses, the estimated response to the mixture is zero (i.e., the mixture is also at a subthreshold dose).

U.S. EPA reports and in several review articles (ATSDR 2018; Boobis et al. 2011; Groten et al. 1996; Martin et al. 2021; Mumtaz and Hertzberg 1993). In general, the information on toxicological interaction varies widely, from subjective comparisons of the toxicity associated with individual components to that of the mixture, to statistical evaluations (modeling and significance testing) that compare the dose-additive predicted response with the observed mixture response. Historically, most evaluations of interactions have involved binary mixtures. Occasionally, studies have described mechanistic understanding of the key interaction events (ATSDR 2004). Some reviews of toxicological interaction have concluded that much of the supporting evidence for interactions occurs at fairly high exposure levels (Cedergreen 2014) and includes high-dose properties like kinetic saturation or change(s) in toxicodynamics (El-Masri et al. 1996). For the no-interaction versions of both dose addition and independent action (response addition or effect summation), U.S. EPA's recommendation has been to apply them when exposure levels are low enough that non-specific toxicological interaction is not likely (U.S. EPA 2000c). When interactions (deviations from dose addition) are likely or potentially significant, U.S. EPA's interactions-based HI, discussed in Section 2.2.1.2, is recommended when data/information are available to estimate the parameters in the formula.

2.1.7. Recent Publications Evaluating Dose Addition

Some in vivo studies have examined predicted mixture responses based on dose-addition models for specific groups of chemicals (Altenburger et al. 2000; Conley et al. 2023; Conley et al. 2022; Crofton et al. 2005; Gennings et al. 2004; Hass et al. 2017; Howdeshell et al. 2015; Kortenkamp and Haas 2009; Martin et al. 2021; Moser et al. 2005; Moser et al. 2012; Mwanza et al. 2012; Rider et al. 2008; 2010; Rider et al. 2009; U.S. EPA 2007b; Walker et al. 2005). Those studies mainly focused on whether the experimentally observed responses were consistent with predictions of a dose-additive model. For a review of recent articles on statistical and experimental design issues for dose-addition analyses, see Rider and Simmons (2018), primarily Chapters 9, 11, and 13. While some of these studies examined groups of compounds that are thought to target the same toxicodynamic pathways (Moser et al. 2012; Mwanza et al. 2012; Walker et al. 2005), others examined compounds thought to target different toxicodynamic pathways but lead to the same health outcome (Rider et al. 2009). Generally, the results of these studies support the continued application of dose addition as U.S. EPA's default approach for mixtures of toxicologically similar chemicals, particularly in the low-response region of a mixture dose-response function; however, some of these studies have identified issues with the interpretation of "toxicological similarity" and "independence" in relation to the use of dose-additive approaches for prediction of mixture risk. In general, the scope of data scenarios to which the assumption of dose additivity is appropriate has been expanded to include some of

those considered previously to suggest independence and the use of response addition. These issues, and others, related to grouping chemicals based on evidence of toxicological similarity are discussed in Section 3.

Several biostatistical approaches have been developed to evaluate the predictions of dose-addition models. When U.S. EPA published its 2000 Supplementary Chemical Mixtures Guidance, which included dose addition based on Finney's similarity model (Finney 1942), the model developed by Berenbaum (1985)[12] was increasingly being used widely for examining whether predicted/modeled health outcomes deviated from dose additivity. Since then, several additional or modified methods have been published (Altenburger et al. 2000; Gennings et al. 2004; Hertzberg et al. 2013; Howard and Webster 2009; Jonker et al. 2005; Olmstead and LeBlanc 2005; Rider et al. 2009). These methods generally are targeted applications or modifications of the Berenbaum method or modifications of the relative potency approach developed by Finney (1971). Each of these methods detect departures from dose additivity and can be used both to estimate the relative potency of specific components and to predict responses to chemical mixture exposures. Some key mathematical aspects of these topics are discussed in Section 4.

2.2. U.S. EPA RISK ASSESSMENT APPROACHES BASED ON DOSE ADDITION

In practice, U.S. EPA applies dose addition in environmental risk assessment to estimate response to a mixture when component dose-response information is either the only or the best information available and when there is a common toxic effect associated with the component chemicals. U.S. EPA uses two basic methods based on dose addition: the HI and RPF approaches (U.S. EPA 2000c). Other methods, mainly statistical, have been published that can be used to analyze toxicological data on the component chemicals and the whole mixture to determine empirical support for dose additivity in the evaluation of joint toxicity of mixtures, or to predict the mixture response in the dose-response assessment stage of the risk assessment, and. These methods often use the Berenbaum equation (Berenbaum 1981). This section summarizes the methods contained in the U.S. EPA (2000c) mixture risk assessment guidance, as well as other methods developed by U.S. EPA programs and offices that extend those concepts. More extensive discussions of the above approaches are provided in Section 4, with additional concepts described in Section 5.

[12]Berenbaum's equation is the isobole formula originally published by Loewe and Muischnek (1926).

2.2.1. Hazard Index Approaches

Among the component-based risk assessment methods, the simplest and perhaps most widely used is the HI.[13] The HI is particularly useful in that it is applied in the risk characterization step of a risk assessment, where estimated population exposures are combined with toxicity (i.e., hazard) information to characterize the potential for adverse health effects associated with a mixture of chemicals. This contrasts with most dose addition approaches, which are applied as part of the dose-response assessment step. The HI is a decision aid and has been shown to be useful in chemical mixtures decision contexts. The HI and all of the HI variants and refinements (discussed in this section) that consider target organ, route, and other factors are not expressed as a probability, nor are they expressed as an estimate of a toxicity measure (e.g., percentage decrement in enzyme activity).[14]

In the HI approach, a hazard quotient (HQ) is calculated as the ratio of human exposure (*E*) to a human health reference value (RfV)[15] for each mixture component chemical (*i*) (U.S. EPA 1986). These HQs are summed to yield the HI for the mixture, as in Eq 2-5.

$$HI = \sum_{i=1}^{n} HQ_i = \sum_{i=1}^{n} \frac{E_i}{RfV_i} \qquad (2\text{-}5)$$

In human health risk assessments, using the RfV approach, U.S. EPA's preferred values for noncancer effects are the reference dose (RfD; for oral and sometimes dermal exposure routes) and the reference concentration (RfC; for the inhalation exposure route).[16] When those are not available, alternatives include other U.S. EPA values or similar exposure duration-specific values derived by other agencies, such as the Agency for Toxic Substances and Disease Registry (ATSDR) Minimal Risk Risk Level (MRL) (ATSDR 2018). Because the

[13]U.S. EPA defines the HI as a weighted sum of the exposure measures for the mixture component chemicals. Because the HI is based on dose addition, the "weight" ideally provides an estimate of the component chemical's toxicity, relative to those of the other components. See additional discussion on page 79 of U.S. EPA (2000c).
[14]Some describe the HI as an indicator of potential hazard because it does not estimate the probability of an effect; others characterize the HI as an indicator of potential risk because the measure integrates both exposure and toxicity (U.S. EPA 2000c).
[15]U.S. EPA (2002c) states "the term reference value is used here generically to refer to values such as the RfD, RfC, acute reference exposure (ARE), Health Advisory (HA), acute exposure guideline level (AEGL), minimal risk level (MRL), or other similar values."
[16]U.S. EPA (IRIS Glossary) defines an RfD as "an estimate, with uncertainty spanning perhaps an order of magnitude, of a daily oral exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime." Similarly, U.S. EPA (IRIS Glossary) defines an RfC as "an estimate (with uncertainty spanning perhaps an order of magnitude) of a continuous inhalation exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime. It can be derived from a NOAEL, lowest-observed-adverse-effect level (LOAEL), or benchmark concentration, with uncertainty factors (UFs) generally applied to reflect limitations of the data used. Generally used in U.S. EPA's noncancer health assessments. [Durations include acute, short-term, subchronic, and chronic…]."

numerator of each HQ is typically the estimated human exposure, the human health values used in the denominator must correspond and be a human toxicity RfV, either derived directly from human epidemiological studies (e.g., effective dose [ED_x] from benchmark dose modeling) or derived from human-equivalent values converted from animal studies (e.g., application of cross-species dosimetric adjustments to points of departure to account for potential differences in toxicokinetics between animals and humans and then application of an animal-to-human uncertainty factor [UF_A]).

An HI less than or equal to one (1) is regarded as being of minor or no concern to human health, typically requiring no further analysis (U.S. EPA 1986; 1991a; 2000c). An HI greater than one (1) is generally regarded as being of possible concern for human health. For these situations, further evaluations may be undertaken to better understand the potential for health effects associated with exposures to the individual chemicals and their contributions to the joint toxicity associated with the mixture.

The HI has important characteristics to consider in its application. It is based on dose addition (Svendsgaard and Hertzberg 1994; U.S. EPA 2000c), with which the formula in Eq 2-5 is consistent. With dose addition, the mixture exposure is the potency-weighted sum of the component exposures (see Section 2.2.2 for more discussion of dose-addition formulas). The HI formula is consistent with that concept in that the toxic potency is represented by the inverse of an RfV (U.S. EPA 1991a; 2000c). Thus, instead of $\beta_1 d_1$ in Eq 2-2 for Chemical 1, the term is $(1/RfD_1)d_1$. Furthermore, HI is dimensionless, so in the HI formula, E and the RfV must be in the same units. For example, if E is the oral intake rate (mg/kg-day), then the RfV could be the RfD, which has the same units. If E is a concentration in an environmental medium such as a drinking water concentration (mg/L), the E term would need to be transformed (based on the ingestion rate, exposure frequency, exposure duration, and body weight) to an estimate of the daily intake dose in mg/kg-day for comparison with the RfD. Alternatively, if E is characterized as a concentration, the RfV would need to be converted to an equivalent media-specific value (e.g., health-based water concentration). Similarly, inhalation exposures measured in or estimated to occur in ambient air (e.g., ppm or ppb) would need to be converted to an equivalent concentration in mg/m^3, as the RfV would usually be the RfC, which is typically expressed in units of mg/m^3. (Consultation with the appropriate U.S. EPA programs, offices, and regions is suggested for identification of recommended practices).

One advantage of the HI formula in risk communication is that interpretation of the results is relatively straightforward. One limitation of the HI is that comparisons of HI estimates across different exposure routes/scenarios (e.g., oral versus inhalation; comparing drinking water HIs to soil ingestion HIs) can be misleading and challenging to interpret. Because the HI is based on dose addition, it implies that if two exposure scenarios involve the same chemicals and their

HI values are the same, then with other factors being equal (e.g., exposure frequency and duration; similar health endpoints; similar lifestage), the two exposure scenarios could be judged to have the same potential for causing toxic effects. That interpretation has the strongest scientific foundation when there are only minor differences in the component exposures (thus, same exposure route, same chemicals, similar exposure duration for specific receptors) between the two scenarios. Comparative interpretation is more difficult if there is greater uncertainty associated with exposure or with the toxicity value for a chemical for one route or scenario (e.g., oral ingestion) versus another (e.g., inhalation), or if there are differences in the chemical compositions of the mixtures. For example, an exposure estimate or prediction for a mixture chemical via one exposure pathway may come with greater variability or uncertainty than another exposure pathway that is better informed by available data. Likewise, a toxicity value for one exposure route may come with greater quantitative uncertainty (i.e., RfV was derived using a large composite UF) than a toxicity value for the same chemical via another exposure route. In general, greater uncertainties in the exposure and/or toxicity value(s) for a given mixture chemical will often cause a corresponding route-specific HQ to increase. In summary, comparison of multiple HIs is strongly situation and data specific, and not merely a simple comparison of their numerical values.

The HI represented by Eq 2-5 (denoted HI_{ALL} in Section 2.2.1.1) includes all chemicals that are in the combination exposure regardless of the precise endpoint or effect on which each individual RfV is based. Because each component RfV represents that chemical's overall critical effect, this summing of HQs is usually considered health-protective for environmental exposures, e.g., synergistic interactions are deemed unlikely. When the component RfVs represent different target organs, the formula may not closely follow the assumption in dose addition of toxicological similarity because the dose scaling factors (1/RfV) are not based on the same (shared) effect. A related uncertainty with application of the HI_{ALL} is that a given chemical might have a different critical effect for different exposure routes and/or durations. These issues have been addressed by different formulas and guidance and are presented in the following sections.

2.2.1.1. Target-Organ-Based Hazard Indices

The HI is based on dose addition with its underlying concept of toxicological similarity. One complication is that the data available on target organs of concern could vary across the chemicals. When the mixture includes chemicals with different critical effects, thus RfVs based on different toxic effects and target organs, the toxicological support for the HI in Eq 2-5 is lower on the continuum of evidence for similarity. A common alternative approach is to develop an HI based on a common target organ. To facilitate comparison among HI and HI variant

formulas, this HI will be renamed HI_{ALL} to denote this "all effects" HI, shown with the example in Eq 2-6.

An alternative approach is to develop an HI based on a common target organ. One variant HI application is to have the HI formula focus on a particular target organ of concern and have the formula's parts be based on toxicity data for that organ. The formula used by U.S. EPA, shown with the example in Eq 2-7, is also called the target organ-specific hazard index (TOSHI) and includes in the assessment group those chemicals whose RfVs are based on effects on that target organ (U.S. EPA 2001b; 2009a).[17] When a chemical shows effects on a target organ but the RfV is based on another effect, the ATSDR calculates a surrogate RfV called the target-organ toxicity dose (TTD) that only uses toxicity data on that target organ (Mumtaz et al. 1997). U.S. EPA (2000c) recommends the development of TTDs. U.S. EPA's IRIS Program recently began to report organ-specific RfVs (i.e., TTDs) for some human health assessments (U.S. EPA 2012). Risk assessors should coordinate with the relevant U.S. EPA program, office, or region regarding the HI methods used.

To allow broad(er) application of the effect-specific HI formula, the chemicals included may not be required to have the same MOA but only the same target organ or apical effect (U.S. EPA 1991a; 1999; 2009a). A chemical's target organ is defined here as an organ or organ system that is adversely affected by exposure to the chemical. A chemical can affect multiple organs, but the term "target organ" is usually applied to the effects of most concern for a given exposure scenario, and thus, to those usually occurring at lower exposure levels, which include but are not limited to the overall critical effect (i.e., the target organ effect that would underpin the derivation of an RfV that is presumably protective against all other known effects induced by a given chemical). Such target organ approaches are utilized in U.S. EPA hazardous waste site assessments (U.S. EPA 1991a; 2001b). One such approach involves separation of HIs by target organ (i.e., HI_{TO}) and requires identification of the major health effects of each chemical, including those seen at higher doses than those causing the critical effect. For example, the chemical may cause liver damage (the critical effect) at a dose of 10 mg/kg-day and neurotoxicity at a dose of 250 mg/kg-day; this chemical might not be included in the HI_{TO} calculation for neurotoxicity (i.e., this chemical does not have an RfV based on neurotoxicity) but the chemical's neurotoxicity would be noted in the discussion of uncertainties. For a TTD type of HI, this chemical would be included in a neurotoxicity-based HI calculation.

[17]In the TOSHI approach used by U.S. EPA, the assessment group includes chemicals with a critical effect on the same target organ or system. Using this approach, some chemicals affecting the same target organ, but without a critical effect in that target organ, might be excluded, potentially resulting in the exclusion of other target organs only seen at doses higher than the critical effect dose. Consequently, health hazards and health risks may be underestimated.

These variants of Eq 2-5 are presented and compared below, distinguished by subscripts. The comparison begins with the approach described in the U.S. EPA guidance for hazardous waste site assessments (U.S. EPA 1991a; 2001b). An HI calculation can be performed using all mixture chemicals regardless of their target organs. As noted above, HI_{ALL} denotes this "all effects" HI. To be more consistent with the concept of toxicological similarity, the first refinement to this HI_{ALL} is the TOSHI, which includes those mixture chemicals that affect the specified target organ of interest and have their RfVs based on that target organ effect. Let HI_{TO} denote this target-organ-specific HI, where "TO" is replaced by the target organ (e.g., HI_{LIV} for when the HI only includes chemicals with RfVs based on the liver effects). To allow inclusion of chemicals that affect the target organ of interest but that have an RfV for a different effect, the second refinement substitutes the TTD in place of the RfV; this ensures the HI formula has all potency scaling factors based on that target organ effect of interest. Let HI_{TTD} denote this TTD-based HI. The ATSDR has developed the TTD as the RfV equivalent by using data on the target organ of interest but otherwise following their procedure for estimating acceptable levels such as the ATSDR MRL (Mumtaz et al. 1997). In practice, such a TTD would usually be higher than that chemical's overall RfV based on the critical effect. The three HI variants are now summarized.

HI_{ALL}: Includes all chemicals, and all terms use the critical effect-based RfV in the denominator, likely resulting in a collection of different critical effects. This HI_{ALL} is used, for example, for assessing hazardous waste sites.

HI_{TO}: Restricted to chemicals affecting the same target organ of interest and whose critical effect is based on toxicity in that target organ. Denominators then use each chemical's critical effect RfV for the specified target organ.

HI_{TTD}: Restricted to chemicals affecting the same target organ of interest. Denominators use a TTD instead of the critical effect-based RfV whenever the critical effect does not impact the target organ of interest; the TTD is only based on data for the target organ of interest. This allows chemicals to be included that are not in the HI_{TO}. By being more inclusive, the HI_{TTD} can reasonably be expected to better characterize the mixture toxicity for the specified target organ than would the HI_{TO}. Incorporating such chemicals in the HI calculation by use of TTDs should be done in coordination with the U.S. EPA program, office, or region.

Now assume that at least one chemical affecting the target organ of interest has a critical effect based on a different target organ. Then the following ordering will occur.

$$HI_{ALL} \geq HI_{TTD} \geq HI_{TO}$$

Note that the toxicological information on the target organs of interest needed to inform application of the HI_{TO} and HI_{TTD} may not be available for all components of environmental mixtures.

Example: HI variants for liver toxicity. Consider exposure to a mixture of Chemicals A, B, and C via oral ingestion with a focus on noncancer toxicity. Examples of various target organs or health effect domains associated with oral exposure to different mixture components are illustrated in Figure 2-2 (U.S. EPA 2023b). Key information for this artificial example is summarized below:

Chemical	Affects Liver?	Critical effect target organ for RfD
A	Yes	Liver
B	Yes	Liver
C	Yes	Kidney (liver effects at higher doses)

HI_{ALL} in Eq 2-6 sums HQs for all three chemicals using each chemical's RfD in the denominator, regardless of the dissimilarity in critical effect. A TOSHI for liver effects, HI_{LIV}, then includes only Chemicals A and B, because Chemical C's critical effect is not in the liver. Thus, for the liver as the target organ of interest, $HI_{LIV} < HI_{ALL}$ because now the HQ for Chemical C is excluded from the sum. The interpretation of this target-organ-based HI is then closer to the toxicological similarity concept of dose addition than is HI_{ALL} because now all of the chemicals included in the calculation share the same target organ of interest (the liver) and the dose scaling factors (1/RfD) are also for that common target organ. Because Chemical C also affects the liver, the TTD-based HI includes Chemical C in the HI calculation by having its liver TTD be in the denominator. Thus $HI_{TTD} > HI_{TO}$. Because the liver is not the critical effect for Chemical C, $TTD_C > RfD_C$ and thus $HI_{ALL} > HI_{TTD}$.

All Effects HI_{ALL}:

$$HI_{ALL} = \frac{E_A}{RfD_A} + \frac{E_B}{RfD_B} + \frac{E_C}{RfD_C} \tag{2-6}$$

Liver-based HI_{TO}:

$$HI_{TO} = \frac{E_A}{RfD_A} + \frac{E_B}{RfD_B} \tag{2-7}$$

Liver-based HI_{TTD}:

$$HI_{TTD} = \frac{E_A}{RfD_A} + \frac{E_B}{RfD_B} + \frac{E_C}{TTD_C} \tag{2-8}$$

In general, each refinement will usually change the quantitative magnitude of the HI. However, the endpoint-specific information needed for the HI_{TO} or HI_{TTD} may make the ability to utilize the refined approaches challenging in many cases. Further, these refined approaches could result in loss of component chemical representation in an HI_{TO} or HI_{TTD} due to lack of evidence for a given health effect of potential concern. In addition, an HI_{TTD}, based on organ- or endpoint-specific toxicity values, will usually be numerically lower than the HI_{ALL} as shown in the above example. These biological and quantitative issues with HI variants typically are addressed when characterizing the sources and magnitude of uncertainty. Although the TTD is not in common use at U.S. EPA, and TTD values are only available for a few chemicals because of the endpoint-specific information needed, the substitution by the TTD can make a significant difference in the value of the HI. Mumtaz et al. (1997) showed some examples with oral exposure in which an organ-specific HQ is reduced by a factor of 10 or more by replacing the RfD by the TTD. Further examples of numerical changes from use of a TTD-based HI, including variations in the data supporting the TTD and possible dominance in the mixture by secondary effects, have been published (Lambert and Lipscomb 2007; Lipscomb et al. 2010; Lipscomb et al. 2013; U.S. EPA 2000c). In its 2012 report to Congress, U.S. EPA announced its plans within the IRIS Program to develop toxicity values for multiple effects associated with the chemical being evaluated (U.S. EPA 2012). To the extent that such planned toxicity values would reflect organ- or effect-specific data, they seem similar in concept to the ATSDR TTD values. While U.S. EPA does not have a program that routinely develops TTD values needed to implement this approach, ATSDR has developed several TTDs as part of their Toxicological Profiles program.

One caution on interpreting these latter formulas is that target-organ-specific applications of the HI, while closer in concept to the toxicological similarity that is the basis of dose addition, are not necessarily health-protective: they apply only to a single target organ of interest, which may not be the most sensitive across all mixture component chemicals. A protective target-organ-based risk characterization for the full mixture exposure then includes evaluation of target-organ HI values for each target organ of interest and associated uncertainties.

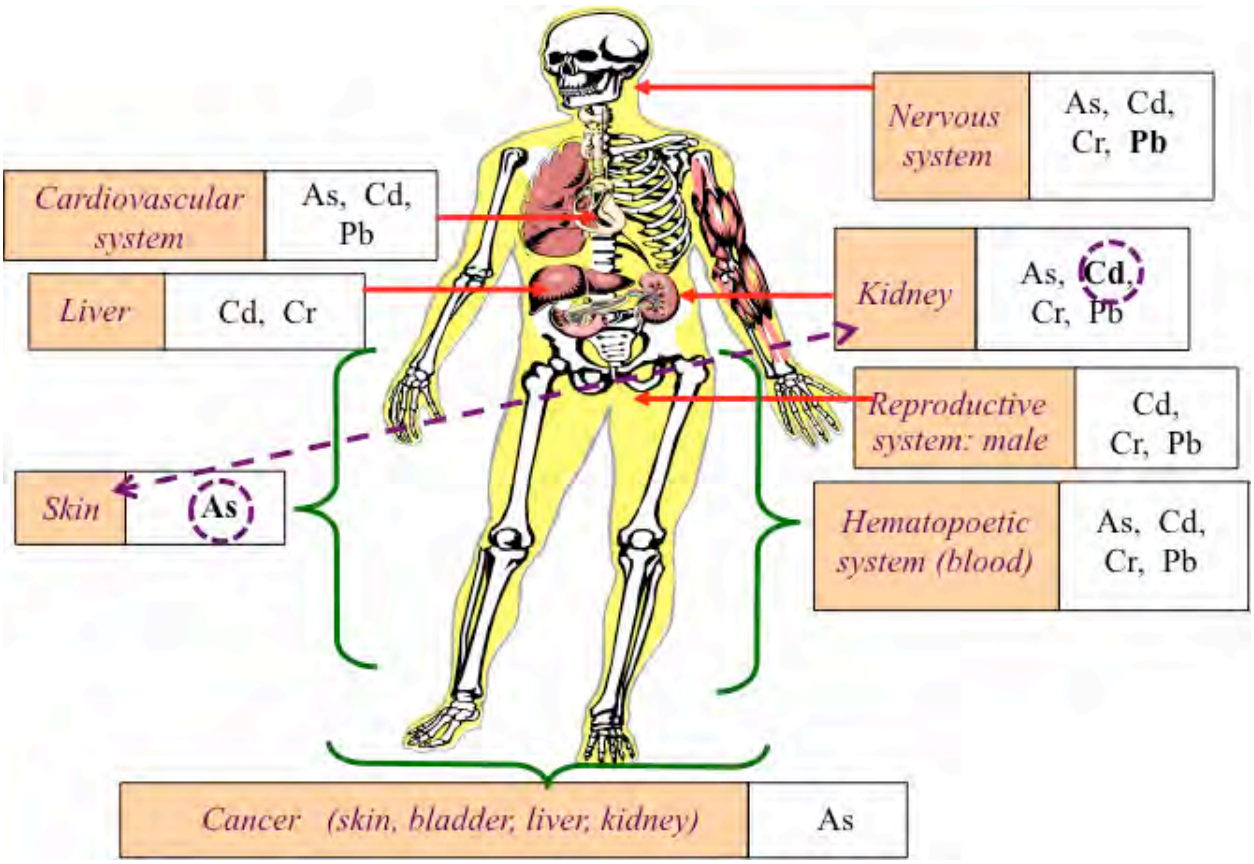


Figure 2-2. Examples with four metals (As, Cd, Cr, Pb) showing multiple adverse effects and differences in critical-effect target organs.

An organ-based oral HI for Cd and As for nervous system effects might use RfDs for the more sensitive kidney and skin effects (dotted circles and line) but would then be an overestimation of nervous system hazard compared to a calculation using nervous system TTDs.
Source: Hertzberg and Mumtaz (2018).
As = arsenic; Cd = cadmium; Cr = chromium; HI = hazard index; Pb = lead; RfD = reference dose; TTD = target-organ toxicity dose.

2.2.1.2. Interaction-Based Hazard Index

As an application of the dose-addition concept, the HI carries an assumption that the included chemicals are toxicologically similar. The HI method also assumes no toxicological interactions (defined by U.S. EPA as departures from additivity), which is partly supported by the decision point at HI = 1, where the estimated component effects are low and interactions are not expected (Mumtaz and Hertzberg 1993). Yet some chemical combinations have strong evidence of interaction, including greater than dose-additive joint toxicity. Not considering such interactions can be a source of uncertainty and possible bias and has raised public concerns about the quality of U.S. EPA's mixture risk assessments. U.S. EPA developed the interaction-based HI (HI_{INT}) as a practical, component-based method that can easily incorporate known information on toxicological interactions. Historically, most experimental interaction studies

involve only pairwise interactions, i.e., those found in binary mixtures (ATSDR 2023; Groten et al. 1996; Mumtaz and Hertzberg 1993), although higher order mixtures are increasingly reported in the scientific literature (e.g. Altenburger et al. 2000; Crofton et al. 2005; Gennings et al. 2004; Jonker et al. 1990; Moser et al. 2012; Olmstead and LeBlanc 2005; Rider et al. 2009; Simmons et al. 2018; Tajima et al. 2002). To incorporate toxicological interactions into a practical approach, U.S. EPA developed a quantitatively modified, interaction-based, hazard index (HI_{INT}) that reflects the potential for pairwise interactions as indicated by the available evidence (U.S. EPA 2000c).

In the formula for the HI_{INT}, each chemical's HQ is multiplied by a factor that captures the magnitudes of all the potential pairwise interactions with the other chemicals.[18]

$$HI_{INT} = \sum_{j=1}^{n} HQ_j \sum_{k \neq j}^{n} f_{jk} M_{jk}^{B_{jk} g_{jk}} \tag{2-9}$$

Each factor in this formula is only summarized here, being described in detail elsewhere (Hertzberg et al. 1999; Hertzberg and Teuschler 2002; U.S. EPA 2000c; 2007a).

M_{jk} = Pairwise interaction magnitude, the ratio of the expected to observed isotoxic dose (e.g., ED_{10}), where "expected" refers to the dose-additive prediction. If that ratio < 1 then the inverse ratio is used so that $M > 1$. U.S. EPA's default assumption is that, for a specified target organ, the binary interaction magnitudes are symmetric, so $M_{jk} = M_{kj}$, and that $M = 5$ unless data show otherwise (see additional discussion below). A further assumption is that M is constant over the dose ranges of interest.

B_{jk} = The binary weight-of-evidence (WOE) score (see below for details). $B < 0$ for less than dose-additive interaction, and $B > 0$ for greater than dose-additive interaction.

f_{jk} = The index of toxic hazard (per its HQ) of Chemical k relative to the total hazard from all chemicals potentially interacting with Chemical j.

g_{jk} = The degree to which Chemicals j and k are present in equitoxic amounts, as indicated by their HQ values.

For greater than dose-additive interactions, interaction magnitude (M) is defined as the ratio of the observed mixture dose to the equitoxic dose predicted by dose addition; for less than dose-additive interactions, it is the inverse ratio (the ratio of the equitoxic dose predicted by dose addition to the observed mixture dose). This definition gives M as the "fold-change" in equitoxic dose. For example, if the binary mixture ED (ED_{10}) predicted by dose addition is 20 mg/kg but

[18]The adjustments are made using data on chemical pairs because the vast majority of studies on toxicological interactions have been conducted on mixtures containing two chemicals.

the actual observed binary mixture ED_{10} is 35 mg/kg, then the mixture is less toxic than predicted by dose addition, and the interaction magnitude, *M*, is 35 ÷ 20 = 1.75. In this case, the mixture is 1.75-fold less toxic than predicted by dose addition. When pairwise interaction magnitude is not available, U.S. EPA (2000c) suggested a default value of 5; further research on the interaction magnitude for mixtures at low doses by Boobis et al. (2011) provides limited support for that default value. The formula in Eq 2-10 has desirable properties and bounding characteristics. For example, if the components are at equitoxic levels (same HQ values) with the same interaction magnitude, the HI_{INT} formula is simply M^B times the HI (Hertzberg and Teuschler 2002). Continuing the above less-than-dose-additive example (assuming excellent data so $B = -1$), if exposures for a different binary mixture of the same two chemicals, but at differing exposure levels and relative proportions, led to HI = 2, then the HI_{INT} is $2 \times (1.75)^{-1} = 2 \div 1.75 = 1.14$, where that smaller result reflects the less-than-dose-additive interaction. For more example calculations using the HI_{INT} formula, see Section 4.3.1.7 of U.S. EPA (2000c). U.S. EPA (2000c) recommends that "all component-based quantitative mixture risk assessments should be limited to one significant digit for the risk value, unless substantial justification is given for higher precision." Consult the relevant U.S. EPA program, office, or region for consideration of the number of significant digits to include in HI calculations and expression of the HI.

Quantitative estimates of interaction magnitude are rare (Boobis et al. 2011); thus, a practical approach is a WOE evaluation of the potential for interactions among the components in the mixture (Mumtaz and Durkin 1992). The WOE approach in the U.S. EPA (2000c) guidance is structured, but simple, involving four categories in which numerical scores are based on the degree of toxicological understanding of the interaction and the extent of extrapolation required to apply the results to human health risk assessment (see Table 2-1). U.S. EPA uses a default value of 0 for the WOE score when interaction evidence is inadequate (see Categories IV-A and IV-B), but also uses a score of 0 when evidence is good that dose addition (no toxicological interaction) is likely. When dose addition is a default mixture assessment approach because of inadequate information, that uncertainty should be described. In the same vein, each pairwise interaction magnitude *M* in Eq 2-10 can be assigned a default value whenever measured interaction magnitudes are not available. For example, U.S. EPA (2000c) identified a value of 5 for the default interaction magnitude.[19] The magnitude *M* (in Eq 2-9) is then modified by the WOE score for that chemical pair. Other WOE approaches could also be

[19]In their analysis of the literature on "low-dose synergy" (a term defined in the article), Boobis et al. (2011) identified six scientific papers (initially over 90 mixtures studies were identified) that met the authors' screening criteria and estimated the magnitude of the interaction. In these studies, the magnitude of synergy at low doses ranged from 1.5 to 3.5. This limited evidence supports a factor of 5 as a conservative, but plausible, upper-end estimate of this parameter.

used (U.S. EPA 2007a). ATSDR, for example, has developed a structured WOE approach for evaluating the evidence on binary interactions relevant to a mixture assessment (ATSDR 2018). The main difference from U.S. EPA's approach is that ATSDR's categorization scheme is more toxicologically detailed and based on three groups of data characteristics: mechanistic understanding, toxicological significance, and other modifiers when further extrapolation is needed (e.g., in vitro data). The two WOE schemes are similar, however, with both based on rough consensus among mixture toxicologists and risk assessors about the strength of evidence and the extent of extrapolation to human toxicity. Either could be used with the U.S. EPA HI_{INT} formula. The WOE factors can be developed ad hoc or, if available, can be taken from ATSDR interaction profiles (ATSDR 2023). As of August 2023, ATSDR had posted 12 interaction profiles as final and 3 as draft. A 2009 summary of the ATSDR mixtures program noted that the interaction profiles and related ATSDR mixture documents available at that time reflected 380 evaluations of binary interactions between chemicals for toxic endpoints of concern (Pohl et al. 2009).

Table 2-1. U.S. Environmental Protection Agency Weight-of-Evidence Categories and Scores for Chemical Interactions[a]

		Direction	
Category	**Description**	**Greater than additive**	**Less than additive**
I	The interaction has been shown to be relevant to human health effects and the direction of the interaction is unequivocal.	1.0	−1.0
II	The direction of the interaction has been demonstrated in vivo in an appropriate animal model, and the relevance to potential human health effects is likely.	0.75	−0.5
III	An interaction in a particular direction is plausible, but the evidence supporting the interaction and its relevance to human health effects is weak.	0.5	0.0
IV	The assumption of additivity has been demonstrated or must be accepted, because the information is one of the following: • Insufficient to determine the direction of any potential interaction. • Insufficient to determine whether any interaction would occur. • Adequate as evidence that no toxicological interaction between/among the compounds is plausible.	0.0	0.0

[a]Adapted from Tables 4-3 and 4-4 of U.S. EPA (2000c). The description for Category IV has been revised for clarity.

The interpretation of HI_{INT} is like that of HI: if $HI_{INT} < 1$, then no further action is deemed necessary. The application of this index requires some information on at least binary

interactions. While simple, such information is rarely available. Consequently, there are limited data sets available for testing its adequacy, and its use in U.S. EPA mixture assessments has been rare. It has been included in the scientific literature as a useful tool for evaluating the impacts of environmental mixture exposures. Lin et al. (2017) used the HI_{INT} to evaluate the interaction effects of As, zinc (Zn), and copper (Cu) in metal-contaminated fish in Taiwan coastal areas, and Omrane et al. (2018) included use of the HI_{INT} in their occupational study research proposal to evaluate interactions of heavy metals. Marx et al. (2015) explored environmental exposures to antibiotic mixtures and used the HI_{INT} to quantify increased risks above those generated using dose-addition. Haddad et al. (2001) calculated an HI_{INT} for systemic toxicants using data on tissue doses of the mixture constituents. Based on the work of Haddad et al. (2001), the health risk assessment of trihalomethane mixtures from reclaimed water during toilet flushing in China was conducted using the HI_{INT} for noncancer effects (Niu et al. 2015). Ryker and Small (2008) applied the HI_{INT} to evaluate cardiovascular and neurological interaction effects from exposures to mixtures of As, Cd, and manganese (Mn) in drinking water as a method for identifying priorities for drinking water research. Finally, Kumari and Kumar (2020) reviewed several component-based approaches for mixture risk assessment and identified the EPA HI_{INT} approach as the most appropriate for predicting joint toxicity of chemical mixtures. They also demonstrated the HI_{INT} applicability and challenges using emerging contaminants as an example. Since 2000, further work on interactions and ways to evaluate interaction evidence has led to variations on HI_{INT}, for example, use of toxicokinetic interaction models to estimate actual tissue doses (ATSDR 2018; Haddad et al. 2001; U.S. EPA 2007a).

2.2.1.3. Multiroute/Multipathway Hazard Index

Many environmental exposures involve more than one exposure route or pathway, such as inhalation of contaminated urban air along with ingestion of contaminated drinking water. A modification of the HI, i.e., the multiroute hazard index (MHI), addresses such exposures. In Agency guidance for hazardous waste sites, this MHI can be calculated by summing HIs across all the exposure pathways using route-specific RfVs (U.S. EPA 1991a). ATSDR has also employed the MHI approach for assessing multipathway exposures at hazardous waste sites (ATSDR 2005; ATSDR 2018; Pohl et al. 2009; U.S. EPA 1991a) and has a framework for estimating total integrated exposure across multiple routes or media (Mumtaz et al. 1995). U.S. EPA also has developed guidance for multiroute (aggregate) exposure assessments for individual pesticides, with explanations of why both route and pathway need to be considered for exposure assessment and why the RfVs need to match the exposure routes when evaluating risk and hazard (U.S. EPA 2001a). The remainder of this section focuses on an extension of the HI

formula to address multiple exposure routes. In this report, route and pathway have the following definitions (ATSDR 2005; U.S. EPA 2001b):

- *Route*: How people physically contact the environmental chemical (i.e., inhalation, ingestion, or dermal contact).
- *Pathway*: The course a chemical takes from the source or release point to the exposed individual, reflecting the release pattern, environmental fate and transport processes, exposure point or area, exposure route, and the exposed population.

Extending the HI to address multiple routes begins with defining a route-specific HQ. When there are *m* routes and *n* chemicals, the HQ for the j^{th} chemical by the k^{th} route is:

$$HQ_{jk} = \frac{E_{jk}}{RfV_{jk}} \tag{2-10}$$

The k^{th} route-specific HI for *n* chemicals is the HI restricted to exposures by that route:

$$HI_k = \sum_{j=1}^{n} HQ_{jk} \tag{2-11}$$

The MHI for *m* routes is then the sum of the *m* route-specific HIs.

$$MHI = \sum_{k=1}^{m} HI_k \tag{2-12}$$

The same numerical result will be achieved if the MHI is calculated by first summing across routes (obtaining an aggregate HQ for each chemical), and then summing across the *n* chemicals.

$$MHI = \sum_{j=1}^{n} \left(\sum_{k=1}^{m} HQ_{jk} \right) \tag{2-13}$$

Eqs 2-12 and 2-13 differ in the intermediate information. Eqs 2-10 and 2-11 help identify which route is of most concern, while Eq 2-13 helps identify which chemical is of most concern.

Example. Consider the hypothetical scenario of four chemicals and three exposure pathways for a community: inhaling volatile compounds released from the water while showering, ingesting fish, and drinking water, where the fish, drinking water, and showering water come from the same source water. The scenario involves two exposure routes: oral intake

and inhalation. The assessment then involves four chemical-specific aggregate HQs and two route-specific HIs, which can be used to identify key exposure routes and chemicals for potential evaluation in regulatory risk management decisions. As shown in Table 2-2, the intermediate calculations of the HIs show more significant digits than one, as is good mathematical practice for intermediate calculations. The calculation of MHI is 4.5, the primary route of concern is oral (drinking water and ingesting fish) with oral HI = 4.1 (3.5 + 0.57), the primary medium of concern is contaminated tap water with the multiroute tap water HI = 3.9 (= 3.0 + 0.5 + 0.44), and the primary chemical of potential concern is Chemical D (oral HQ = 3.0). If the example had not included Chemical D (or if its concentration were much lower), the MHI would still exceed 1 because of the other three chemicals. In this example, the MHI of 4.5 could be rounded to one significant digit (i.e., MHI = 5) because the noncancer toxicity RfVs are expressed as one significant digit (U.S. EPA 1991a; 1991b; 2000b; 2000c); HI expression with additional significant digits may be appropriate in some circumstances, depending on the underlying data and the relevant procedures provided by the U.S. EPA program, office, or region. While the example uses the HI_{ALL}, the multiroute HI could be organ specific and thus use HI_{TO} or HI_{TTD}.

Table 2-2. Estimated Hazard Quotients and Hazard Indexes for Hypothetical Residential Exposures to Chemicals from a Single Water Source[a]

Exposure route, contaminated media, and chemicals[a]	Chemical exposure level	Noncancer toxicity RfV[b]	Chemical and route-specific HQs[c]	Multiroute HQ (Chemical C)	Media-specific HIs	Route-specific HIs
Ingestion (oral)	**mg/kg-d**					
Fish						
Chemical A	3.5×10^{-5}	1×10^{-4}	0.35		0.57	
Chemical B	4.4×10^{-6}	2×10^{-5}	0.22			
Tap water						4.1
Chemical C	1.0×10^{-2}	2×10^{-2}	0.50		3.5	
Chemical D	1.2×10^{-2}	4×10^{-3}	3.0			
Inhalation	**mg/m^3**			0.94		
Indoor air (showering)						
Chemical C	8.7×10^{-3}	2×10^{-2}	0.44			
Chemical D	1.0×10^{-2}	Not established	—		0.44	0.44
Total multiroute HI *(summed across chemicals, media, and exposure routes)*						***4.5*** **(rounds *to 5)***[d]

[a]Adapted from MacDonell et al. (2018).

[b]The toxicity RfVs are for chronic exposures similar to RfD and RfC values in U.S. EPA's IRIS (U.S. EPA 2023b). Dose additivity is assumed for this HI estimate. The calculated HQ and HI values are presented to two significant figures.

[c]A dash (—) indicates the value is not calculated (because an inhalation RfC has not been established for Chemical D).

[d]U.S. EPA (2000c) recommends that "all component-based quantitative mixture risk assessments should be limited to one significant digit for the risk value, unless substantial justification is given for higher precision." Consult the relevant U.S. EPA program, office, or region for consideration of the number of significant digits to include in HI calculations and expression of the HI.

HI = hazard index; HQ = hazard quotient; IRIS = Integrated Risk Information System; RfC = reference concentration; RfD = reference dose; RfV = reference value.

2.2.1.4. Uncertainties with Hazard Index Approaches

Important uncertainties exist with using and interpreting the HI. First, the HI is a decision index and not a probabilistic risk estimate; a doubling of the HI does not necessarily imply a doubling of the risk of adverse health effects. For this reason, much of the interpretation is left to the risk practitioner.

A second uncertainty involves the use of RfVs, that is, the denominators in Eq 2-5. Specifically, application of UFs in high dependence of RfV derivation on chemical-specific data

considerations (e.g., source of a given point of departure [POD; human or experimental animal]; cross-species kinetics and dynamics; exposure duration used in a principal study). As such, the magnitude of a given HQ, and a corresponding HI, may be greatly influenced by RfVs with large composite uncertainties. There are many sources of RfVs, including RfD and RfC values that are available online at https://www.epa.gov/iris; these terms are defined in Footnotes 13 and 14. Peer-reviewed RfVs are generally preferred; for specific applications of the HI, relevant program office guidance should be sought. Because reference values are based on each chemical's critical effect, an HI using Eq 2-5 is a health-protective approach that could overestimate the hazard when used in an assessment in which the critical effects used to derive the RfVs across mixture chemicals differ. RfVs like the RfD and RfC are estimates of human exposures that are "likely to be without an appreciable risk of deleterious effects." They are not sharp estimates of a toxicity threshold. Use of 1/RfD or 1/RfC in a chemical's HQ is thus considered to be health-protective, giving a likely overestimate of that chemical's potency scaling factor. The potential overestimate increases the confidence of a minimal hazard when HI ≤ 1. For example, use of RfVs in the HQs could be appropriate in applications that establish a health-protective level at which no known or anticipated adverse effects are expected to occur. If the HI > 1 then there may be health risks. Where sufficiently robust information is available, further evaluations may be undertaken of the various uncertainties to identify risk drivers, including those chemicals with the highest HQs, which may clarify overall uncertainty.

A third uncertainty is due to the variability of the toxicological databases that underlie RfVs. While the dose-addition concept scales doses by the same quantity (e.g., ED_{20}), the chemical components could have health-based RfVs that differ in terms of the margin between their exposure levels and exposures at which effects might be expected, and thus represent different potential for hazard. For example, the RfC for Chemical A might be based on chronic-exposure human toxicity data while the RfC for Chemical B could be derived from subchronic-duration studies using mice, thus, introducing uncertainties related to extrapolation across species and exposure durations.

The HI is derived from dose addition; therefore, the uncertainties that relate to the extent of toxicological evidence and various assumptions related to the dose-addition concept of similarity of toxic action also apply to the HI. Mixture chemicals could affect the same general target organ profile but have different critical effects (i.e., most sensitive effect among the profile of effects). Chemicals might influence each other's internal concentration but mixture data to show that influence could be missing. The same chemical might have a different critical effect for different exposure routes, increasing the uncertainty in any multiroute HI application. A related uncertainty for exposure assessment is that chemicals in different media might have different speciation (e.g., metals) or bioavailability or have different time-dependent fate and

transport processes in or through those media. These differences imply changes in the mixture composition and dose at the target population. If these uncertainties are not accounted for correctly, the mixture toxicity characterization, potential interactions, and resulting risk estimates could be substantially in error. Many of the toxicological uncertainties might be better understood as information on MOA or factors that could affect tissue exposure (e.g., deposition pattern in the nose for nasal lesions) becomes available. Similarity of toxic action is discussed in greater detail in Section 4.

2.2.2. Relative Potency Factor Approaches

RPF approaches comprise the second basic dose-addition method used by U.S. EPA in assessments of risks posed by exposures to mixtures. There are two types: the general RPF approach that has been applied to pesticides and a few other chemical groups, and the toxicity equivalency factor (TEF) approach that was originally developed for mixtures of dioxins and dioxin-like compounds (DLCs). The TEF approach is considered a special case of the RPF approach. The general RPF approach implemented by U.S. EPA follows the Finney definition of dose addition, thus estimates of relative potency are assumed to be constant over the dose range of interest.

An RPF is a numerical quantity used to scale the dose of one chemical to an equitoxic dose of another chemical by accounting for differences in their potencies in causing the same/similar health effect; the latter chemical is typically termed the index chemical. The index chemical is usually the chemical with the highest quality or most robust toxicological database in the group or mixture being assessed and the chemical considered to be most representative of the type of toxicity caused by the other mixture components (U.S. EPA 2000c; 2002a; 2002b). Further, the index chemical must have dose-response data for the dose range of interest. In the RPF approach, the assumption under dose additivity is that the toxicity of each component of the mixture "behaves" in accordance with a fixed concentration or dilution of the chemical selected as the index chemical (U.S. EPA 2000c). The RPF is the ratio of the potency of the individual component to that of the index chemical. By definition, the RPF for the index chemical is 1. The potency can be estimated from the response at a fixed dose or the dose for a fixed response. For example, when carcinogenic risk is described by a low-dose linear model, the slope at low doses is roughly constant, so the cancer risk estimate is the slope multiplied by the dose (U.S. EPA 2005a). The potency ratio can then be calculated as the ratio of the low-dose slopes (commonly known as "slope factors" [SFs]) as shown in Eq 2-14:

$$RPF_j = \frac{Slope_j}{Slope_{IC}} \quad (2\text{-}14)$$

where the subscript "IC" refers to the index chemical.

For example, if the SF for Chemical 1 is twice that of the index chemical, then for a given dose, the risk from Chemical 1 will be twice that of the index chemical, and thus Chemical 1 is twice as potent as the index chemical. That is reflected in the calculation: $RPF_1 = 2$. When response-specific doses are used, the inverse ratio is calculated. For example, if using the ED_{10}, then the RPF formula has the index chemical value in the numerator, as shown in Eq 2-15. If Chemical 2 is twice as potent as the index chemical, its ED_{10} will be half as large; thus, ED_{10} for Chemical 2 must be in the denominator.

$$RPF_j = \frac{ED10_{IC}}{ED10_j} \tag{2-15}$$

The doses of the individual components are scaled by the RPFs and then summed to yield the index chemical-equivalent dose (ICED) of the entire mixture:

$$ICED_{MIX} = \sum_{j=1}^{n} d_j * RPF_j \tag{2-16}$$

Presently, U.S. EPA determines a single RPF for the response range or dose range of interest. When data so indicate, a different RPF can be determined for more specific conditions, for example, each effect and each exposure scenario (e.g., hepatotoxicity versus renal toxicity, acute versus chronic exposure, oral versus inhalation exposure). As explained further in the next section, that flexibility or scenario specificity is the main difference between the general RPF application and the more restricted TEF approach.

A numerical assessment of the noncancer health hazard or cancer risk associated with exposure to the mixture is then obtained by using the ICED with common single-chemical methods (e.g., with the index chemical's RfD or RfC to estimate the mixture HQ, or with the index chemical's SF to estimate cancer risk). When a dose-response function exists for the index chemical, the mixture response can be quantitatively estimated directly from the mixture's ICED and the index chemical's dose-response function, preferably based on high-quality dose-response data (U.S. EPA 2016). For example, if the index chemical's dose-response model is denoted $f(d)$, then the RPF-based response to the mixture is estimated as:

$$y_{MIX} = f(ICED_{MIX}) \tag{2-17}$$

where the ICED is from Eq 2-16. The RPF approach is a direct application of dose addition. The result is then not a numerical indicator of concern, as with the HI, but an estimated response to the mixture. Consequently, to justify using the RPF approach, typically stronger evidence

supporting dose addition is required than with the HI [i.e., that the components share a common MOA (U.S. EPA 2000c)]. U.S. EPA's supplementary guidance (U.S. EPA 2000c) also states that the evidence for similarity of toxic action and corresponding RPF application can be restricted: "The common mode-of-action assumption can be met using a surrogate of toxicological similarity, but for specific conditions (endpoint, route, duration)." U.S. EPA has developed RPFs for only a few chemical groups, such as the organophosphorous pesticides, the triazines, the *N*-methyl carbamates, chloroacetanilides, and the pyrethrins/pyrethroids (U.S. EPA 2023a).

2.2.2.1. Toxicity Equivalence Factors

The TEF is mathematically similar to the RPF, and the associated mixture methods are mathematically equivalent, but the TEF is a special case that requires much more information (see Table 2-3). Initially, the term "toxicity equivalence factor" was defined as a consensus, relative toxic potency estimate where a single TEF is assigned to each chemical (U.S. EPA 2000c). The RPF approach using TEFs has thus far been used by U.S. EPA only to evaluate mixtures of dioxins and DLCs. The approach assumes essentially complete similarity so that each component acts as a true dilution or concentration of any other component. The evidence supporting that assumption is that most, if not all, of the biological and toxic effects of the DLCs are mediated through aryl hydrocarbon receptor (AhR) binding, including cancer and noncancer effects (U.S. EPA 2010). That allows one TEF to be calculated for each chemical and applied to any endpoint and response level. Such an interpretation requires a high degree of evidence for toxicological similarity and is thus described as a "special case of the RPF method"[20] (U.S. EPA 2000c; 2010). When a chemical group lacks that degree of similarity, such as when the relative potencies change for different effects, the RPF designation is used, not the TEF. For the dioxins and DLCs, U.S. EPA has recommended application primarily to the oral exposure route, with application to dermal and inhalation routes "as an interim estimate or as a component of the sensitivity analysis..." (U.S. EPA 2010). The TEFs have been applied to multiple endpoints because the endpoints have been shown or are assumed to result from a common initial mechanism of action, that is, the DLC binding to the AhR. This detailed understanding and extent of similarity of toxic action rarely exists, and so TEFs are expected to apply to only a few chemical groups.

[20]U.S. EPA (2010) describes TEFs as a special case of RPFs because TEFs require detailed knowledge about the toxicity mechanism and the extent to which toxicological similarity among a group of chemicals is assumed. TEFs were originally developed as an interim approach for assessments of dioxins, furans, and dioxin-like polychlorinated biphenyls [see U.S. EPA (2010) and citations, particularly those describing the history of TEF development, therein].

Table 2-3. Comparison of Toxicity Equivalency Factors and Relative Potency Factors[a]

Toxicity equivalence factor	Relative potency factor
Specific type of relative potency factor	Generalized case
All health endpoints	May be focused on a specific health endpoint
All routes of exposure as interim estimate	May be focused on a specific exposure route
All life stages	May be focused on a specific life stage
Encompasses all dose ranges	May be focused on a specific dose range
Assumes same mechanism of action underlies all toxicity	Assumes similar mode of action
One toxicity equivalency factor set for all scenarios	Different relative potency factor sets can be used for different scenarios (e.g., to reflect differences in exposure routes)

[a]Adapted from U.S. EPA (2000c) and U.S. EPA (2010).

2.2.2.2. Uncertainties with Relative Potency Factor Methods

The RPF approach is appealing because it closely follows the toxicological similarity concepts of dose addition and because its assumption of constant potency values means it can use Agency RPF values to estimate risk or hazard under the actual exposure conditions for the mixture of concern. Such use of official values also confers some standardization across RPF applications and stability of such assessments over time because those values are slowly updated. When component RPFs are missing, other approaches based on dose additivity can be applied. The main conceptual uncertainty occurs when the assumption is made, without empirical support, that a particular set of RPFs is fixed for multiple scenarios (e.g., differences in dose range, exposure route, exposure duration) and so applies to the scenario being addressed. Exceptions have been noted, even for the more restrictive TEFs. The above assumption was challenged in the data-rich example of the DLCs, where the TEFs were characterized as best applied to oral exposures, with use of the same TEFs for other routes advised only as a needed interim procedure (U.S. EPA 2010). One rarely stated assumption with RPF approaches (or any approach using constant values for relative potency) is similarity of maximal effect. When some components are partial agonists, they cannot have an equivalent dose that produces a more extreme response. Some solutions have been published for addressing partial agonists (Gennings et al. 2004; Howard et al. 2010; Howard and Webster 2009). A simple solution to avoid this problem is to restrict the application of the RPF and similar methods to response ranges below that of the lowest maximal component response. Another uncertainty is that some examples have been published in which the DRCs are not similarly shaped, and so RPFs change with dose or response level (Dinse and Umbach 2011). For example, an RPF based on ED_{10} ratios could be

significantly different from an RPF based on ED_{30} values. Several detailed discussions of this uncertainty along with some possible solutions are presented in Section 4.

2.2.3. Program-Specific Applications of Dose Addition

Several U.S. EPA programs use dose-addition approaches for addressing health risks from exposure to chemical mixtures. In these approaches, the dose-addition concepts in the U.S. EPA guidelines (U.S. EPA 1986; 2000c) have been adapted to fulfill the statutory obligations regarding mixture risk (i.e., they were developed with program-specific regulatory and policy aspects in mind). The program guidance usually builds on the Agency guidelines, discussed in previous sections, in part to address implementation as well as specific scenarios, and data availability issues that are commonly encountered.

The HI approach, as the first example, is used by U.S. EPA for risk characterization[21] of noncarcinogenic health effects from mixtures at hazardous waste sites (U.S. EPA 1991a), in drinking water (U.S. EPA 2000a), and in ambient air (U.S. EPA 2015b). In each case, the HI approach follows the concepts and formulas shown in Section 2.2.1, although the target-organ HI can be applied differently by different U.S. EPA program offices. For addressing hazardous waste sites, U.S. EPA has published specific guidance for the HI to address multiple-pathway exposures (U.S. EPA 2001b) and to simplify the assessment by screening out chemicals judged unlikely to contribute to the overall mixture risk or hazard (U.S. EPA 1991a). The HI approach used by the U.S. EPA Office of Air Quality Planning and Standards is similar to the applications to Superfund sites in that the preferred approach is to calculate a separate HI for each target organ (U.S. EPA 2015b; 2018a).

The margin-of-exposure (MOE) approach, as a second example, is used for risk characterization of multiple pesticides in accord with the Food Quality Protection Act (FQPA).[22] Like the HI, the MOE is not an estimate of health risk (e.g., fraction of exposed population expected to show toxic effects); it is also not an estimate of an expected response (e.g., percent enlargement of liver). The MOE is a decision tool that, in conjunction with the target (benchmark) MOE, indicates how far the estimated human mixture exposure is from an equivalent estimated mixture POD (U.S. EPA 2000a). The pesticide mixture MOE approach begins by identifying those pesticides that share a common toxicological mode or mechanism of

[21]As mentioned previously in Section 2.2.1, the HI is not a predictive risk estimate, but a numerical aid to decision making.

[22]For single chemical assessments, the MOE is the point of departure for that chemical divided by the exposure to the chemical. The target or benchmark MOE is used as a lower decision bound for MOE values, so lower MOE values would suggest an unacceptable hazard. That is similar to the value of 1.0 as an upper bound for the HI, where higher HI values suggest an unacceptable hazard. The target MOE is situation-specific but often is the product of the UFs that would be applied in calculating a reference dose. For example, a target MOE is often 100 if chronic rat studies are used to calculate the MOE so an MOE = 130 would suggest no significant hazard (U.S. EPA 2023a).

action (U.S. EPA 1999). The MOE calculation for a specific exposure route involves estimating RPFs for all pesticides in the common mechanism group (CMG) and calculating the ICED (the sum of the RPF-weighted exposure levels for all pesticides in the group), following the same concepts presented in the earlier Section 2.2.2 on RPFs. The mixture MOE approach then compares the mixture dose (as its ICED) to the route-specific POD of the index chemical (U.S. EPA 2002a; 2007b). The FQPA assessments also reflect an assumption of concurrent exposure to the multiple pesticides that can include multiple exposure routes. Consequently, extensive guidance has been developed for assessing pesticide exposure variations over time and across different environmental media (U.S. EPA 2001a). The resulting "total MOE" (MOE_T) formula for multimedia exposure to the CMG is the inverse of the sum of inverses of the route-specific MOE values [see page 52 of U.S. EPA (2001a) and page 167 of U.S. EPA (2007b)]. For *k* routes of exposure, the total MOE (MOE_T) is:

$$MOE_T = \frac{1}{\frac{1}{MOE_1} + \frac{1}{MOE_2} + \ldots + \frac{1}{MOE_k}} \quad (2\text{-}18)$$

where the calculation of each route-specific MOE is modified to account for differences in UFs across the chemicals [for details on those UF-based modifications for the MOE_T, see U.S. EPA (2001a) and U.S. EPA (2007b)].

Both the HI and MOE approaches produce risk-based numbers that are used in each case as an indicator of the potential for a mixture of components to produce adverse health effects. Many of the program-specific methods are sufficiently robust and general that they could be considered for application to other risk assessment situations involving mixture exposure.

2.2.4. Integrated Addition Approach

An underlying assumption of the dose-addition methods described in this section is that the chemicals in the mixture are toxicologically similar. Many mixture exposures, however, contain component chemicals that are not toxicologically similar or for which information on toxicological similarity does not neatly fall into a single toxicological similarity group. This scenario arose with the evaluation of the feasibility for health risk assessment of drinking water disinfection by-product (DBP) chemicals (Teuschler et al. 2004; U.S. EPA 2003b). That research led to the development of a hybrid additivity approach that incorporated both dose addition and response addition for toxicity endpoints, thus, producing a mixture estimate that is the probabilistic risk of the adverse endpoint of concern. It was originally termed the cumulative RPF approach to reflect its inclusion of multiple chemicals and exposure routes, but is here referred to as "integrated addition" for consistency with similar published methods (Kim et al.

2014; Mwense et al. 2004; Olmstead and LeBlanc 2005; Rider et al. 2009). The application of the integrated addition method to the DBP assessment is provided here as an example (U.S. EPA 2003b).

For chemicals eliciting a common, toxic effect or endpoint, the integrated addition approach begins with separation of the mixture components into dose-additive groups [called subclasses in U.S. EPA (2003b)] based on toxicological similarity. This corresponds to the pesticide risk assessment step of forming CMGs (U.S. EPA 1999). Next, the assumptions of similarity within groups and then of independence across groups are evaluated by examining existing mixture studies for evidence of interactions. Evidence for interactions might come from studies across different levels of biological organization, spanning from MIEs, key intermediate events, to apical effect (i.e., phenotypic health outcome) data. As such, integration of a WOE for potential mixture chemical interactions may include data from NAM and traditional animal bioassay study designs. It should be noted that use of NAM data, for example from in vitro bioactivity assays (e.g., ToxCast or Tox21), for such a purpose necessitates conversion of in vitro concentrations to administered equivalent exposure doses using reverse dosimetry and in vitro-to-in vivo extrapolation. If there are interactions, other mixture assessment methods, such as the interaction-based HI, would be used. If available evidence does not indicate interactions within each toxicity similarity group, the RPF approach is used to estimate the group risk for each grouping. The individual group risks are then combined across all groupings using response addition. As an example, following the identification of an effect of concern, the specific steps outlined for the DBPs in Section 4.1 of U.S. EPA (2003b) are:

- Form toxicological similarity groups based on available information on MOA (e.g., two similarity groups could cause the same effect through different MOAs).
- Estimate absorbed dose to allow combination of multiroute exposures.[23]
- Develop dose-response models for all component chemicals in each similarity group for the effect(s) of concern.
- Select an index chemical for each similarity group.
- Develop RPFs for each similarity group to reflect within-group potency differences.
- Calculate the ICED for each similarity group.
- Calculate each similarity group mixture risk (as a probability) for the common effect(s) using the index chemical dose-response function.
- Estimate the total mixture risk using response addition across the similarity group risk estimates.

[23]Not necessary for single-route exposures.

These steps are demonstrated using the DBP example in the 2003 feasibility report for cancer risk (U.S. EPA 2003b). Using simple classifications, the DBPs were grouped by genotoxic versus nongenotoxic MOAs for cancer (see Table 2-4); the mechanisms of action within each group were assumed to be the same when they were not fully elucidated. Other similarity groups described in the report, but not included here, were formed for developmental toxicity (cardiovascular), developmental toxicity (general, whole organism), and reproductive toxicity (testis, sperm). The full process of estimating each group risk and combining with response addition is captured for cancer risk in Figure 2-3. U.S. EPA (2022b) also used an integrated addition approach to assess cancer risks associated with the aromatic high carbon range fraction of total petroleum hydrocarbons.

Table 2-4. Integrated Addition Example: Disinfectant By-Products Grouped into Subclasses Based on Assumed Common Modes of Action[a]

Genotoxic carcinogens	Nongenotoxic carcinogens
Bromodichloromethane	Dichloroacetic acid
Bromoform	Trichloroacetic acid
Chlorodibromomethane	—[b]

[a]Source: U.S. EPA (2003b). As with the source document, these subclasses are shown here for demonstration only and do not necessarily reflect Agency designations.

[b]A dash (—) indicates no other nongenotoxic carcinogens considered.

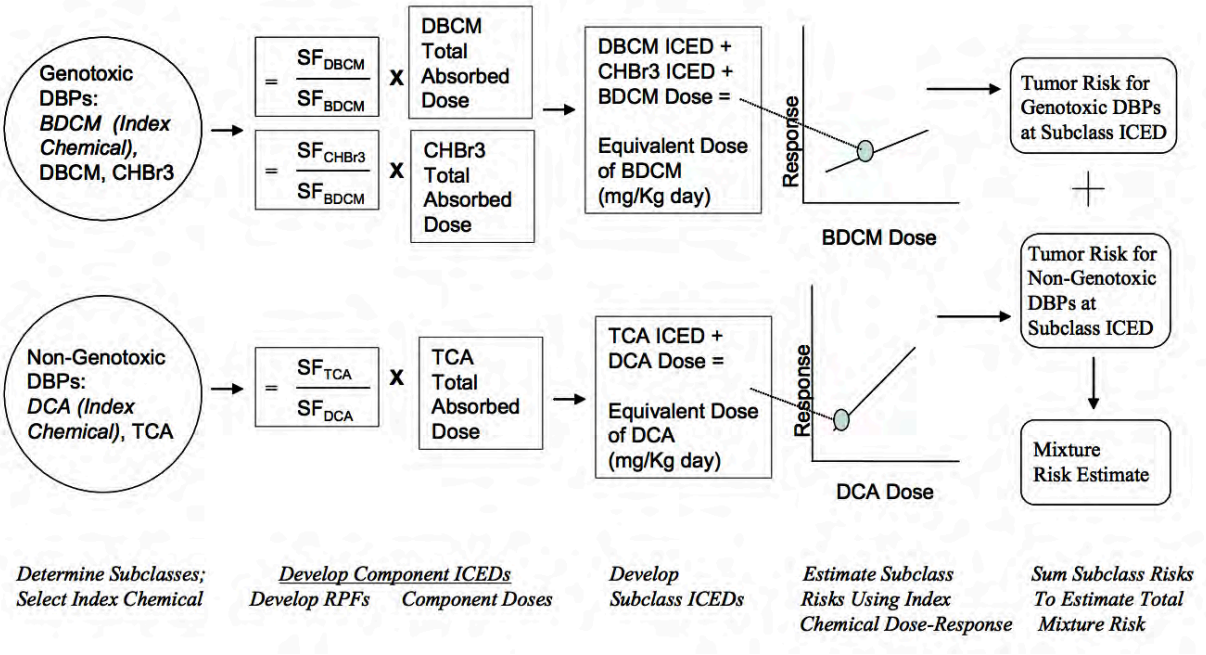


Figure 2-3. Schematic of integrated addition as applied to disinfection by-products divided into two similarity groups.

This illustration is of a cancer risk assessment for a mixture of the following DBPs: BDCM, DBCM, $CHBr_3$, DCA, and TCA. The ratio of the cancer SF for each chemical to that of the index chemical is used to calculate the RPF. [Adapted from U.S. EPA (2003b)]

BDCM = bromodichloromethane; $CHBr_3$ = bromoform; DBCM = chlorodibromomethane; DBP = disinfection by-product; DCA = dichloroacetic acid; ICED = index chemical-equivalent dose; RPF = relative potency factor; SF = slope factor; TCA = trichloroacetic acid.

The RPFs for the DBPs were based on internal human-equivalent dose, so much of the U.S. EPA (2003b) report describes the process of modeling and estimating that dose. This step is needed for multiroute exposure estimation but is not usually done for single-route assessments (see also U.S. EPA 2006a). The use of internal dose may also affect the determination of similarity of shape for DRCs across the mixture components, which may be different from that using administered dose. The determination of similarity of DRC shape will affect the choice of the dose-additive model to apply (see Sections 4.1 and 5.1 for detailed discussion).

In an integrated addition approach, dose addition is applied within each similarity group, but independence holds across groups. For chemicals acting independently, response addition or effect summation is used to estimate mixture risk or hazard, respectively (U.S. EPA 2000c). For integrated addition, examples have applied response addition to quantal (dichotomous) outcomes, where the effect measure is the presence or absence of the effect; the effect metric is then determined in terms of probability or fraction of population affected. Cautions for the use of effect summation are provided in Rider and Simmons (2018). For effects with continuous measures, such as organ weight or enzyme activity, effect summation can be used when

summing across the groups and is best applied when the individual and summed effect magnitudes are small to stay within biological limits on the magnitude of the effect. Continuous measures could also be converted to quantal responses by defining the magnitude of an effect measure that is the boundary for "adverse effects" and then using response addition to estimate the population fraction showing adverse effects. There is, however, the potential for loss of information, such as dose-dependent changes in toxic effects. Several published papers have discussed issues that can arise when performing continuous to quantal response transformation (Dinse and Umbach 2011; Gaylor 1996; Gaylor et al. 1999; Ritz et al. 2006; Slikker et al. 2004).

2.2.5. General Considerations When Using Dose-Additive Models in Risk Assessment

The use of dose-additive models is the default approach of U.S. EPA when conducting health risk assessments associated with exposures to chemical mixtures of toxicologically similar chemicals (U.S. EPA 2000c). Dose-additive models have been mostly used to estimate noncancer hazards but also to estimate cancer risks when the chemicals are similar in terms of carcinogenicity (U.S. EPA 1993; 2010). As with single chemical risk assessments, a mixture health risk assessment includes a discussion of uncertainties. For detailed discussion of uncertainties in forming similarity groups, see Section 3.6. For mixtures, supporting data on the mixture of interest and on component-component interactions are frequently sparse; therefore, many of those uncertainties can only be described qualitatively.

Support for dose addition as a primary component-based approach to mixture risk assessment has been presented in some key publications.[24] The National Research Council (NRC 2013) report assessing pesticide mixture risks to endangered and threatened species commented extensively on dose addition and generally supported the U.S. EPA default assumption of dose addition for chemical mixtures. *The State of the Art Report on Mixture Toxicity* commissioned by the European Union Directorate General for the Environment also reached a similar conclusion regarding the utility of dose additivity as a default assumption for health risk assessments of mixtures of environmental chemicals (Kortenkamp and Haas 2009).

Although dose additivity may be a default approach for many assessments of health risk associated with chemical mixtures, any available evidence, including an assessment of data quality [see U.S. EPA (2000c); Table 2-1], needs to be evaluated carefully early in the assessment process (U.S. EPA 2003b). Further guidance on evaluating data quality is provided in

[24]One report (NRC 2008) endorsed the use of dose addition for chemicals with common adverse outcomes, but recommended against restricting dose addition to chemicals with the same MOA. Some of U.S. EPA's applications of the HI approach are consistent with the NRC recommendations. However, U.S. EPA has not used dose addition calculations (i.e., RPF/TEF methods) to estimate risk when evidence of toxic similarity is limited to a finding of the same target organ, same general type of effect (e.g., liver toxicity), or common adverse outcome, in part due to lack of data supporting such approaches.

U.S. EPA's *Framework for Human Health Risk Assessment to Inform Decision Making* (U.S. EPA 2014a), U.S. EPA's Quality System for Environmental Data and Technology (www.epa.gov/quality), and U.S. EPA's *Peer Review Handbook, 4th Edition* (U.S. EPA 2015a).

The Risk Characterization Handbook (U.S. EPA 2000b), prepared by the Agency's Science Policy Council, describes risk characterization as the step that "integrates information from the preceding components of the risk assessment and synthesizes an overall conclusion about the risk that is complete, informative, and useful for decision makers." To help potential users, this White Paper highlights considerations in presenting the results of risk analyses that rely on dose-additive models, emphasizing the importance of describing underlying assumptions and key uncertainties and highlighting where policy choices were made. The presentation and communication of risk analyses that rely on dose-additive models need to adhere to the overall risk characterization principles of transparency, clarity, consistency, and reasonableness.

The methods described in this section for mixture assessment are based on or derived from dose addition and tend to use an external exposure metric (e.g., ambient air concentration rather than internal dose). Thus, they are simple and relatively easy to implement, requiring only information on exposure and the toxicity of the component chemicals. While the HI and RPF methods can be used with equitoxic doses from any source (e.g., RfD, ED_{10}), there are advantages when these methods are used with established U.S. EPA toxicity values (e.g., RfD/C and RPF) or other U.S. federal agency assessments. For example, because they are official Agency values, they are externally peer reviewed. In general, U.S. EPA risk assessment methods are designed to be more likely to overestimate than underestimate the potential for adverse effects; dose-additive methods are similarly designed. While the HI is an easy to use decision aid, RPF-based predictions can estimate the response to the mixture for specific doses of the mixture components and can be tailored to a specific endpoint or exposure scenario. Further enhancements have been made to these approaches and are discussed in Section 4. Extended methods and more complex concepts about toxicological similarity, including toxicokinetics, toxicodynamics, and use of data from novel in vitro methods, as well as the calculations and uncertainties, are discussed in Sections 3 and 5.

3. CONSIDERATIONS FOR GROUPING CHEMICALS FOR USE IN DOSE ADDITION

Determining which environmental chemicals to include in a group that will be analyzed using the dose-addition methods described in Section 2 (i.e., forming a "dose-additive group") is a necessary step in risk assessments that use such methods. This section identifies considerations that can be useful when evaluating whether potentially relevant chemicals could be included in a dose-additive group. As a default approach for U.S. EPA chemical mixture risk assessments (see discussion in Section 2.2.5), dose addition is applied both across a broad spectrum of chemical mixtures, for which the levels of toxicity and exposure information among the individual component chemicals may vary widely, and across many different situations in which chemical mixtures could be encountered in the environment. Thus, these considerations are intended to encompass this broad spectrum of potential applications. Further, application of these considerations depends on the specific statutory, program-specific, or office-specific requirements prompting the risk assessment.

Depending on the U.S. EPA program or office and the purpose of the mixture risk assessment, two major considerations used by U.S. EPA when evaluating component chemicals for possible inclusion in a chemical group that will be evaluated through a dose-additive approach are:

- **Environmental exposures to the mixture.** In simple cases, environmental exposures to the chemicals would be concurrent or have the potential to result in internal doses that overlap in time. Whether dose addition applies in situations where internal doses do not overlap in time is unclear at present.
- **Similarity of toxic action.** The chemicals elicit or are assumed to elicit a common biological response via a common mode or mechanism of action, a common AOP, or a shared key toxicological event.

3.1. ENVIRONMENTAL EXPOSURE TO MIXTURES

The first consideration helps to simplify the risk assessment for mixtures. In simple cases, for dose addition to apply, environmental exposures[25] to the component chemicals would need to occur within a time frame that results in the overlap of internal doses of the chemicals (note: this could include parent mixture component chemicals and/or associated bioactive transformation products including metabolites). Furthermore, for an accurate assessment, an estimate of the effective internal exposures (e.g., area under the curve; clearance; plasma half-life) for all

[25]U.S. EPA (2019) "Guidelines for Human Exposure Assessment" defines exposure as "the contact of an agent with an external boundary of a receptor (exposure surface). For exposure to occur, the agent and receptor need to come together in both space and time."

chemicals would be needed to account for temporality of elimination following cessation of exposure. Modeling approaches for intermittent exposures are the subject of current and future investigation and will not be covered in this document[26].

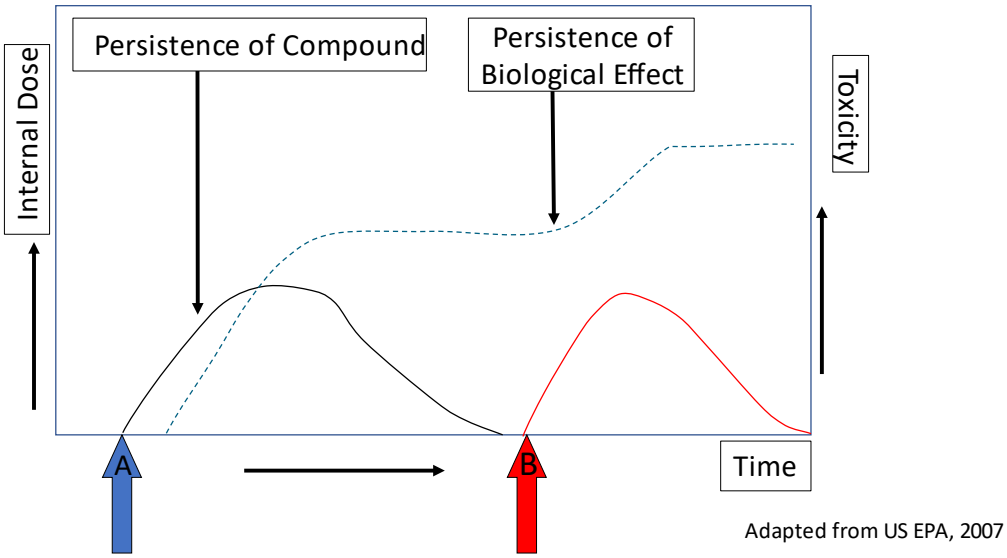
Here, in the context of occurrence of chemicals as a mixture, U.S. EPA distinguishes between time overlap of exposure and time overlap of effects. In Figure 3-1 the *x*-axis represents temporal progression from left to right, spanning initial absorption, time to maximum internal dose (i.e., T_{max}), and eventual elimination. To illustrate the distinction between the time overlap of exposure and time overlap of effects, there are two *y*-axes. The left hand *y*-axis of Figure 3-1 represents internal chemical dose (increases from bottom to top and shown as a solid line) following exposure to the chemical (depicted as wide arrows below the *x*-axis). The right hand *y*-axis of Figure 3-1 illustrates the persistence of the common biological effect (shown as a dashed line following exposure). In Figure 3-1a, the internal doses of Chemicals A and B do not overlap in time, but the effect of Chemical A persists temporally such that exposure to a subsequent chemical ("B") impacts the magnitude and/or incidence of the same/similar effect. At the biological level, in the case of nonoverlapping exposures, there is no pre-existing "dose" (internal concentration) to add to the next exposure, and conceptually, another mathematical model (e.g., response addition or a biologically based model) would apply rather than a dose-addition model. It should be noted that there is potential for imprecise estimation of mixture response in this scenario in Figure 3-1a as the magnitude or incidence of the biological effect is not necessarily dependent on the co-existence of the "doses" of component chemicals. As illustrated in Figure 3-1 (a and b), the persistence of a biological effect is not contingent upon the physical persistence of Chemical A at the target tissue/site. As such, upon subsequent exposure to Chemical B, the tissue is still in a state of biological perturbation. However, the important nuance is that in the scenario presented in Figure 3-1a, toxicological response based on the presence (i.e., dose) of Chemical A or B alone may then be overestimated, even though the response is an amalgamation of the effect(s) of Chemicals A + B. Conversely, in Figure 3-1b, under an assumption of dose addition, the estimation of mixture response for the binary mixture may be more accurately approximated since the doses of Chemicals A and B overlap. In the case of overlapping exposures, a measure of the internal concentration(s) would be needed for an accurate estimate of the effective dose (ED) depending on the time interval between exposures and the elimination kinetics of the chemical(s) in the body. Because data pertaining to internal temporal relationship(s) among mixtures of chemicals (that are encountered in the environment)

[26]Meek et al. (2011) developed a framework that relies on successive tiers. With each successive tier, additional exposure information is required, resulting in assessments that feature increasingly accurate estimates of exposure and decreased uncertainty. Successive tiers are implemented in the Meek et al. (2011) framework when warranted based on a toxicological point of departure.

in the phenotypic expression of toxicity are rarely available, simplifying assumptions are typically made as to the interpretation of mixture dose-responses (e.g., exposure dose as a single point in time, without consideration of internal kinetics/target-site dosimetry, and some toxic effect).

If chemicals occur in the same environmental medium (e.g., food products) at the same time and if people contact the medium (e.g., food consumption), then it could be reasonably assumed that the doses of the chemicals would overlap in time. Similarly, if the chemicals occur in different environmental media at the same time and these media are all routinely contacted (e.g., household air and drinking water), then, again, it could be reasonably assumed that the doses of the chemicals overlap in time. On the other hand, if the chemicals occur in different environmental media at different times and these media are not routinely contacted (e.g., soils in a contaminated waste site and surface waters not near the same populations), then it could be reasonably assumed that the doses of the chemicals would not overlap in time. If chemical occurrence data either in human tissues (e.g., biomarkers of chemical exposure) or in environmental media (along with human contact information) are not available, then human exposure models and environmental fate and transport models for chemicals could be useful in addressing this issue of overlapping chemical exposures (U.S. EPA 2002a).

a)



b)

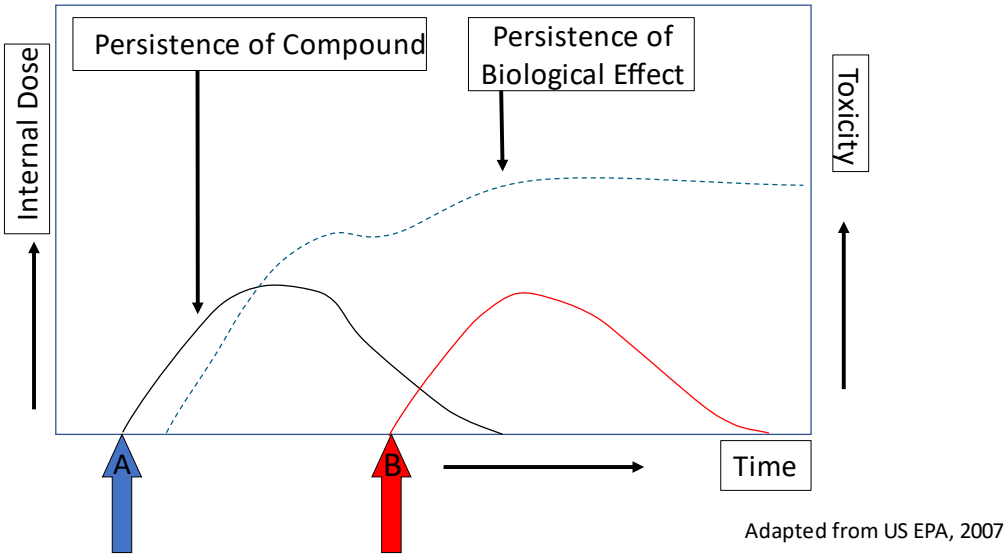


Figure 3-1. Biological effect from exposure to two hypothetical chemicals (A and B) that are toxicodynamic clones a) without and b) with overlap in exposure.

Persistence of the chemicals in the body as internal dose (left-hand *y*-axis) is shown as a solid line following exposure to the chemical (wide arrow below the *x*-axis), and persistence of the common biological effect (right-hand *y*-axis) is shown as a dashed line following single exposures at different times: (a) time overlap of effects only, where exposure to Chemical B occurs after Chemical A has been eliminated from the body; (b) dose overlap, with residual body burden of Chemical A at the time of exposure to Chemical B. This figure illustrates only two examples of many possible scenarios for two chemicals "co-occurring" and is not comprehensive.

3.2. SIMILARITY OF TOXIC ACTION

While U.S. EPA uses methods based on dose addition as a default (previously discussed in Section 2.2.5) and assumes toxicological similarity of the mixture components, confidence in assessments that rely on dose-additive methods is strengthened if some degree of similarity of toxicokinetic and/or toxicodynamic action is demonstrated among the chemicals in the mixture being evaluated. The evidence that can be used to evaluate the similarity of toxic action can vary widely along a continuum from chemicals exhibiting similar structures, physicochemical properties, or comparable kinetics, to those known to affect the same target organ through the same toxicodynamic pathways.

Over the past 20 years, the toxicology community has developed several constructs for assembling and arranging biological evidence along a source-to-health outcome continuum. These include the mechanism or mode of action (MOA), toxicity pathway (TP), and more recently, adverse outcome pathway (AOP). Chemical-specific MOA(s) or TPs (NRC 2007) can be theoretically thought of as detailed constructs under the AOP concept. This process includes the identification of mixture chemical events (e.g., changes in absorption and biotransformation) that precede distribution to systemic circulation and/or target tissue(s) (e.g., first-pass metabolism), molecular initiating events (MIEs; an interaction between the toxic substance and an organism, e.g., binding of a substance to a receptor), intermediate key events (KEs; events that characterize the progression of toxicity following the MIE; KEs are causally linked to one another, and the response-response relationships between successive KEs can be formally assessed using weight-of-evidence analyses), and apical effect/adverse outcomes

Text Box 3-1. Similar Apical Effects

The similar apical effect (i.e., common adverse health outcome) could be applicable to chemicals that induce the "same" specific effect or a similar or shared effect within a "syndrome" of effects resulting from the same dose-additive process (key event). Examples of the former are liver necrosis, thyroid hypertrophy, or decreased serum testosterone. Examples of effect syndromes might include: the profiles of effects associated with neurotoxicity of Type I and Type II pyrethroids (U.S. EPA 2011); the phthalate syndrome (Apel et al. 2020; Kortenkamp and Koch 2020), which entails an overlapping suite of male reproductive effects, such as reduced anogenital distance, hypospadias, epididymal agenesis, undescended testes, and retained nipples, among others, as a result of androgen insufficiency from exposure to endocrine disruptors (Rider et al. 2008; 2010); and "toxic hepatopathy" resulting from exposure to dioxins including hepatocyte hypertrophy, diffuse fatty change, necrosis, portal fibrosis, bile duct hyperplasia, and cholangiofibrosis, among others (NTP 2006a; 2006b; 2006c; 2006d). In the case of multiple related dichotomous effects, the modeled incidence would be based on the appearance of any one of the related endpoints.

(AOs). Each approach is associated with different levels of biological detail, certainty, and/or chemical specificity, although the field has not settled on a systematic comparison of these approaches. For more details regarding the relatedness of the approaches, see Section 5.1.

Some aspects of the possible evidence for similarity are illustrated in Figure 3-2, Figure 3-3, and Figure 3-4. Combinations of evidence can be integrated to evaluate similarity of toxic action. For example, chemical clones (acting exactly alike in the body) would exhibit the same toxicokinetic properties, interact with biological targets through the same MIE, elicit the same sequence of KEs, and result in the same apical health outcome.[27] Chemicals with unknown MIE but with other key toxicokinetic and/or toxicodynamic processes that are similar (or exhibit structural similarity) or chemicals that have the same apical effect or effect syndrome might be placed in a dose-additive group. The evidence for similar toxic action (including kinetics and/or dynamics) can be listed along a continuum. From most to least informative based on similarity, a plausible listing of the evidence is as follows:

a) Same toxic action:[28] Same MIE and subsequent downstream biochemical and biophysical processes (kinetic and/or dynamic KEs), culminating in the same apical health outcome (effect) or effect syndrome (see Text Box 3-1 and Figure 3-2).
b) Similar (but not identical) toxic action: Different MIEs with a common shared downstream biochemical/biophysical process (i.e., a kinetic and/or dynamic KE), where the chemical "doses" add together (i.e., a dose-additive event); after the convergence, the shared KE(s) culminate(s) in a specific apical health effect or effect syndrome (see Figure 3-4 Chemicals D and E, for an example).

[27]Both MOA and AOP (among others) are applicable to this discussion. U.S. EPA (2005a) described MOA as a series of key events and processes starting with interaction of an agent with a cell and proceeding through operational and anatomical changes that cause disease formation. A key point is that the MOA starts with the interaction of the chemical agent and the cell. Ankley et al. (2010) defined AOP as "a conceptual construct that portrays existing knowledge concerning the linkage between a direct MIE (e.g., a molecular interaction between a xenobiotic and a specific biomolecule) and an adverse outcome at a biological level of organization relevant to risk assessment. As such, AOPs are generally a sequential series of events that, by definition, span multiple levels of biological organization." Similarly, the Organisation for Economic Co-operation and Development (OECD) (2013) defined AOPs as "an analytical construct that describes a sequential chain of causally linked events at different levels of biological organisation that lead to an adverse health or ecotoxicological effect." Because the agent itself is not part of the AOP definitions, this section will use the term MOA but will make use of information developed for AOPs in several hypothetical examples. In this context, the term "apical" refers to an observable outcome typically in a whole organism, (e.g., a clinical measure or pathologic state resulting from operational and anatomical changes) that is indicative of toxicity.

[28]Toxicodynamics is defined here and on the Integrated Risk Information System (IRIS) online (U.S. EPA 2023b) as follows: "the determination and quantification of the sequence of events at the cellular and molecular levels leading to a toxic response to an environmental agent (sometimes referred to as pharmacodynamics)." This definition does not include toxicokinetics, which can be different for the mixture components without contraindicating the use of dose-addition methods (see Section 4 for a detailed discussion of this issue). Toxicokinetics is defined as "the determination and quantification of the time course of absorption, distribution, biotransformation, and excretion of chemicals (sometimes referred to as pharmacokinetics)" (U.S. EPA 2023b).

c) Same apical effect or effect syndrome: MIE and other key toxicokinetic and/or toxicodynamic processes unknown.
d) Same target organ.
e) Similar chemical structure: Implied action at the same MIE and implied similar toxicokinetics and/or toxicodynamics.
f) Similar dose-response curve (DRC) shape[29]: Examples of curve similarity are when all components show evidence of dose threshold, or all show a linear (versus S-shaped) curve. Implied proportional toxicodynamics and proportional toxicokinetics, across the component chemicals.

Within each type of evidence listed above, clearly there can be gradations.

3.3. CONSIDERATIONS FOR GROUPING CHEMICALS FOR USE IN ADDITIVE APPROACHES

U.S. EPA (2000c) states, "two chemicals are dose additive if Chemical B is functionally a clone of Chemical A. In the ideal case, the chemicals are assumed to act similarly in terms of … toxicological processes." Figure 3-2 depicts a hypothetical, and relatively simple MOA; referred to as such because this example assumes that the pathway from MIE to AO is well-characterized and systematically evaluated (e.g., use of Bradford Hill criteria). The mixture of concern consists of three chemicals that act as toxicodynamic clones leading to an adverse health outcome through the same MOA. In this depiction, each chemical exhibits the same toxicokinetics and initiates the MOA via the same MIE, culminating in the same AO. The state of knowledge regarding these hypothetical chemicals and the resulting hypothetical MIE, intermediate KEs, and AOs would therefore be complete and the MOA well defined. The model depicted in Figure 3-2 is one of simple similar action, in which there is a common key process by which the chemical concentrations themselves "add together" to elicit a response, acting as dilutions of one another.[30] Each of these three chemicals would be placed in the same dose-additive group based on the evidence depicted in Figure 3-2; confidence in this grouping decision will reflect the well-defined state of knowledge about the shared MOA.

Figure 3-3 illustrates a different state of knowledge about TPs for two environmental chemicals—the situation where the pathway is only partially known and thus characterization of a defined MOA is commonly precluded. Specifically, the scenario for Figure 3-3 assumes that there is knowledge of a shared KE in the toxicodynamic pathway and that perturbing this KE is

[29]Note that the converse does not apply; different curve shapes do not necessarily suggest dissimilar toxicodynamics, so that further research is usually indicated (see Section 4 for further discussion of this issue).
[30]That is, acting as if they were the same chemical, but in different proportions, as described by Bliss (1939).

necessary to elicit the apical outcome. Both chemicals are known to perturb the KE. Whether the chemicals act via the same or different MIEs, or if there are shared intermediate KEs before the common KE, is unknown. There are data demonstrating that exposure to Chemical Y results in the AO, but there are no experimental data on whether exposure to Chemical Z is associated with the AO. With this limited information, the two chemicals might still be placed in the same dose-additive group based on an assumption that Chemical Z would cause the AO due to its known association with the shared/common KE, and inference(s) pertaining to the potency for Chemical Z to induce the AO would be informed by response-response comparisons between Chemicals Y and Z at the level of the common KE. However, because there are inherent uncertainties in qualitatively linking nonapical perturbations (e.g., those measured in new approach methodologies [NAMs]-based assays) and adverse health outcomes, the analyst's confidence in this assumption and the sensitivity of the results to the inclusion or exclusion of Chemical Z in the dose-additive group would be important to discuss both during the risk assessment generally and during the risk characterization specifically (e.g., what is the quantitative uncertainty associated with the uncertainty in the grouping decision). Note that rapid scientific advances are being made in elucidating MOAs and AOPs (Carvaillo et al. 2019; Pittman et al. 2018; Pollesch et al. 2019); the risk assessor will need to take care to distinguish between widely accepted and established MOAs/AOPs and those that are plausible or proposed, and how the pathway-based evidence status across mixture components supports identification of MOA(s) (i.e., relatively full characterization of a chemical-specific pathway) in some instances and AOP(s) in others.

Figure 3-4 depicts a hypothetical chemical mixture that exhibits more complex AOPs exhibiting both pathway convergence and pathway independence. In the example, even though the three chemicals themselves may not be intuitively dose-additive based on apparent independence of action at the level of the MIE, knowledge of the common downstream process(es) suggests that it could be appropriate to group Chemicals D and E. This is one possible hypothetical pathway that could lead to a "common adverse health outcome" as a consequence of exposure to a mixture of chemicals exhibiting convergent AOPs; see discussion in Chapter 5 of NRC (2008). The pathway for Chemical F is seemingly toxicologically independent of the pathway for Chemicals D and E. Chemical F would generally not be included in the dose-additive group with Chemicals D and E (U.S. EPA 1986; 2000c). Rather, the joint toxicity of Chemicals D, E, and F would be estimated using the integrated addition approach (U.S. EPA 2003b), as the elicitation of a health effect in this hypothetical example might entail both elements of dose addition (pathways D and E) and response addition (pathway F). Nelms et al. (2018) illustrated how AOP information can guide the risk assessor to dose-additive or response-additive methods. An important nuance in this example is that in application, the nature

of the “AO” matters; that is, if the AO is a discrete (singular) tissue/organ effect (e.g., liver necrosis) that is influenced by dissimilar proximal KEs across operant pathways then it might be logical to use integrated addition for the example depicted in Figure 3-4. If however the AO entails a constellation or syndrome of related effects that are dependent on a common critical KE (e.g., developmental health outcomes in male progeny resulting from androgen insufficiency such as hypospadias, cryptorchidism, and decreased anogenital distance) then the “AO” at which similarity among mixture component chemicals is determined may need to be evaluated at the level of the common critical KE (e.g., decreased fetal/neonatal androgen levels).

If, based on evidence demonstrating pathway signaling as depicted in Figure 3-4 (different MIE but common key intermediate process), the analyst decides to include Chemicals D and E in a dose-additive group, the decision would be based, in part, on considering toxicological similarity at a greater level of detail than that described in U.S. EPA (2000c). U.S. EPA (2000c) did not address consideration of aspects of joint toxicity at the level of convergent MOA(s), such as common key intermediate processes for chemicals acting via independent MIEs. The example in Figure 3-4 considers application of the dose-additive approach to encompass mixture components that exhibit different initial primary events (e.g., MIEs) but eventually merge in subsequent steps at a common key process/event leading to the AO. A critical consideration in the concept of pathway convergence is that the manner in which mixture component chemicals potentially impact joint toxicity may be multifactorial. For example, in the illustrative example in Figure 3-4, convergence of the pathway for mixture Chemical E with the pathway for Chemical D may entail: (1) the same/a common KE; or (2) kinetic perturbation of one or more key event relationships (KERs) that could potentially impact a response-response relationship in the converged pathway. In the former case (common KE), pathway signal amplification may be a result of the relative contribution of pathways D and E to the shared/same KE in the converged MOA/AOP. In the latter case, there also may be pathway signal modification; however, this could be an amplification or inhibition of signaling dependent on the nature of the kinetic interaction. In many cases, however, the toxicodynamics or kinetics of mixture chemical interactions along a source to outcome continuum will not be well understood. While the chemicals elicit the same effect in a target organ, there may be uncertainty regarding the specific step(s) in a pathway where chemical doses “add” or how response pathways converge. In these cases, the Agency usually assumes, as a default, that the chemicals are dose-additive, placing the chemicals in the same dose-additive group (see Discussion in Section 2.2.5). Consistent with the Agency’s risk assessment guidance, all risk assessment assumptions, including those discussed here in regards to grouping for dose-additive approaches, should be clearly identified and the associated uncertainties characterized.

Conceptually, the assumption of dose additivity does not hold in cases where it can be shown that the chemicals being considered for grouping affect response pathways that are completely independent (no KEs in common). Independent action is illustrated in Figure 3-4 for Chemicals D and F for the AO, as the two pathways elicit the same AO but do not merge. The 1986 Chemical Mixtures Guidelines established, and the 2000(c) Supplementary Chemical Mixtures Guidance supported, the use of independent action methods for chemicals that act via independent MOAs but cause a common health effect (see Section 2.1). Chemicals D and E, however, could be grouped together for dose addition for the AO, because they share KEs in the MOA, even though the initial processes (MIE_1 and MIE_2) are different.

The grouping process is further complicated when information on MOA pathways is missing or uncertain. This topic is addressed also in Section 4.1.4. An experimental result inconsistent with the independence scenario (i.e., Chemicals D and F) depicted in Figure 3-4 is that of Rider et al. (2010), who tested a mixture of dibutyl phthalate and 2,3,7,8-tetrachlorodibenzodioxin (TCDD) for effects on reproductive development in fetal male rats and reported a toxic response that was greater than response additive. That study was conducted in response to the expressed need for "study of combined effects of TCDD, polychlorinated biphenyls (PCBs), and other antiandrogens" as discussed by NRC (2008); however, an evaluation of the consistency of the experimental data with that expected under dose additivity was not possible.

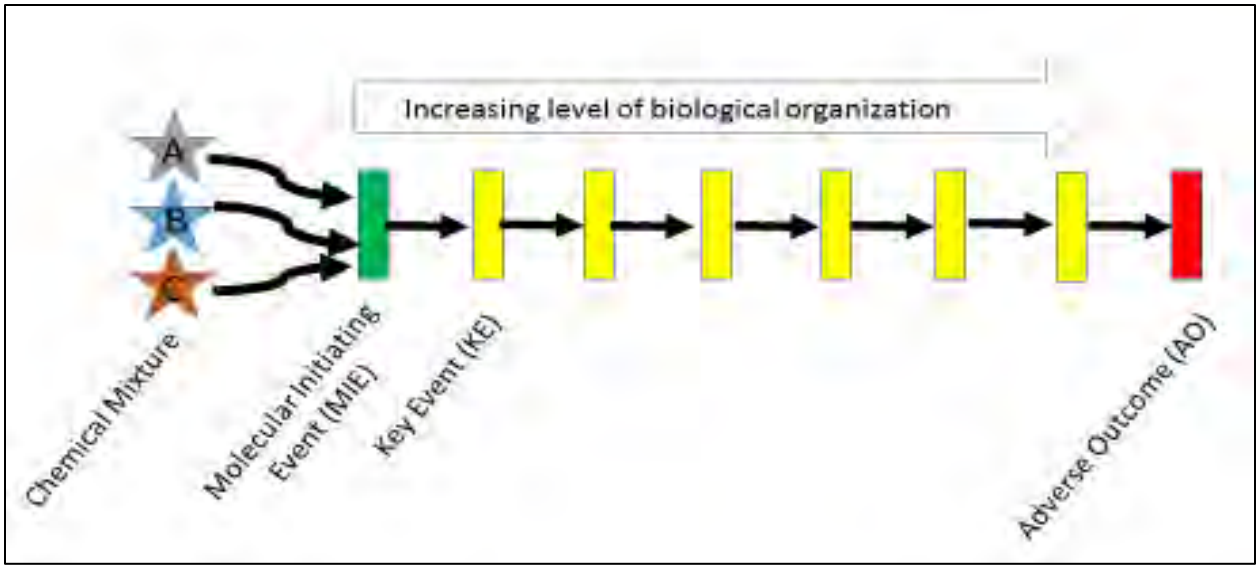
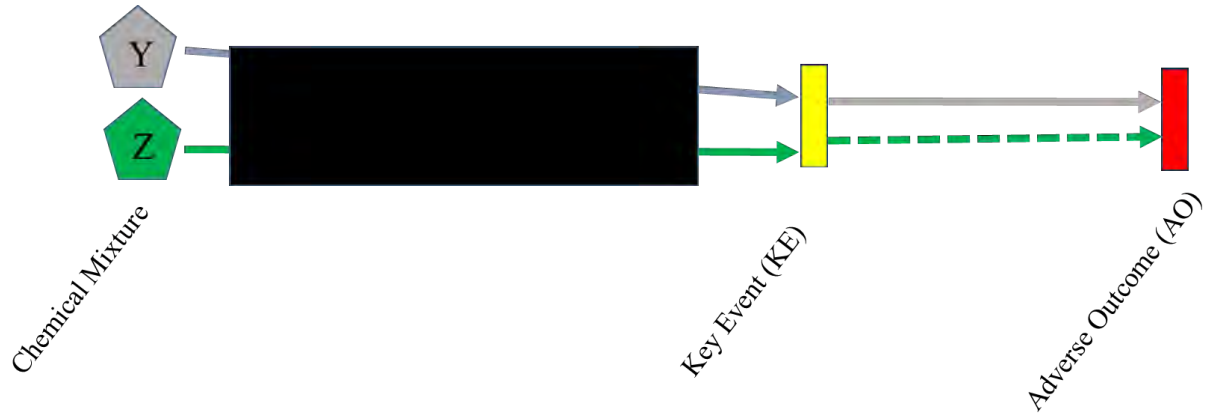


Figure 3-2. A common mode of action that is shared by a mixture of chemicals that act as toxicodynamic "clones" affecting an adverse outcome.

This diagram depicts a hypothetical and relatively simple MOA with one MIE (depicted as a green rectangle), which leads to a linear series of KEs (depicted as yellow rectangles). A mixture of three chemical stressors that act as toxicodynamic clones (symbolized by different colored stars; i.e., Chemicals A, B, and C) comprise a single dose-additive group that perturbs a biological function via a common molecular-level interaction (e.g., binding to a receptor, inhibition of an enzyme, or damage to DNA). The paths of the chemicals to this initial toxicodynamic event are symbolized by different curved arrows to indicate potentially different toxicokinetic processes or differences in the way the chemicals perturb the MIE. Each chemical depicted has a specificity and affinity to the MIE that is the first biological "domino" in the MOA sequence. The initial perturbation can cause additional biological dominos to fall in sequence, where each domino represents a KE at increasing levels of biological organization, finally reaching the observed AO. Each KE can be observed/measured and the progression toward the AO (depicted as a red rectangle) observed. The KERs (straight arrows in Figure 3-2) describe the conditions under which a particular biological change, represented as a KE, will trigger the next KE in the sequence.
AO = adverse outcome; DNA = deoxyribonucleic acid; KE = key event; KER = key event relationship; MOA = mode of action; MIE = molecular initiating event.



Adapted from US EPA, 2017

Figure 3-3. A hypothetical mode of action and a mixture of two chemicals with varying levels of toxicodynamic knowledge.

There is limited information about the MOA, likely including unknown steps that are depicted as a black box. Specifically, one dose-additive KE (depicted as a yellow rectangle) has been identified as a preceding and necessary step that occurs prior to the AO (depicted as a red rectangle). Both chemicals (Y and Z) perturb the KE. Experimental data show that exposure to Chemical Y causes the AO (indicated by solid gray arrows). Due to the lack of data, it is unknown whether Chemical Z by itself causes the AO (i.e., the uncertainty is depicted using the dashed green arrow).
AO = adverse outcome; KE = key event; MOA = mode of action.

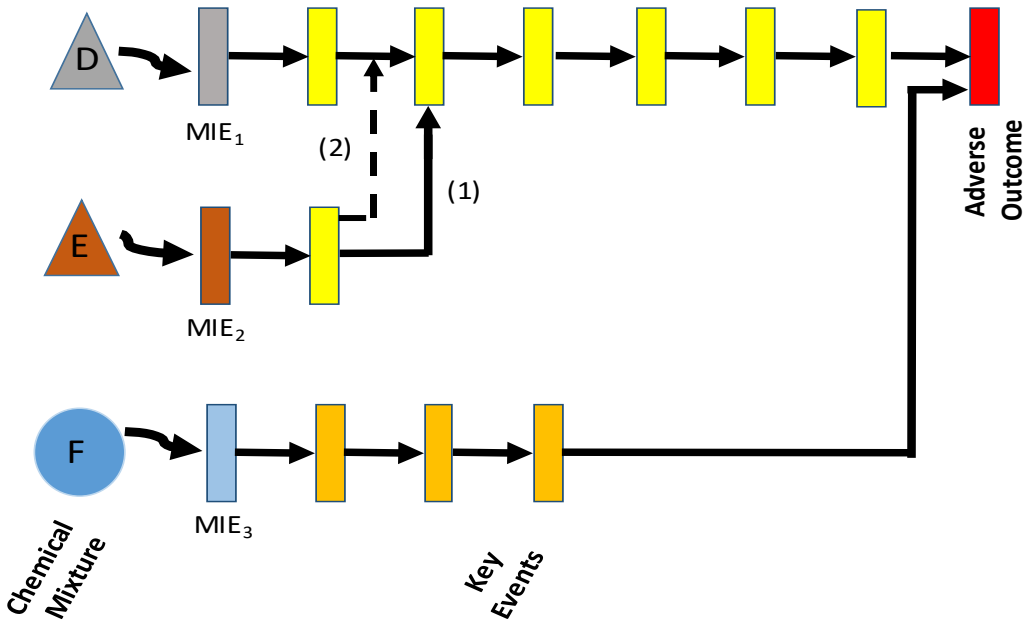


Figure 3-4. Diagram of hypothetical mode of action for a chemical mixture depicting pathway convergence (Chemicals D and E) and pathway independence (Chemical F).

Chemicals D, E, and F affect the AO through three different MIEs. The pathway of KERs (depicted using black arrows) linking MIE_3 to the AO does not intersect the series of KER associated with the pathways from MIE_1 or MIE_2 to the AO (i.e., the pathway initiated by MIE_3 does not have a KE in common with the pathways associated with MIE_1 and MIE_2). The gray and brown triangles and blue circle depict different chemicals in a mixture. The chemicals specifically perturb the MIE of the same color. The convergence of the pathway for Chemical E to Chemical D may occur via one or more events/processes: (1) intermediate KE_1 for pathway E may be the same/similar toxicodynamic event as intermediate KE_2 in pathway D, resulting in amplification of the KE signal; and/or (2) intermediate KE_1 for pathway E may impinge kinetically on a KER in pathway D such that the signaling at a subsequent KE, or beyond, is modified (may be amplification or inhibition), thus potentially impacting the overall AO response.

AO = adverse outcome; KE = key event; KER = key event relationship; MIE = molecular initiating event.

The decision to include or exclude chemicals in a specific dose-additive group ultimately depends on where the strength and breadth of evidence supporting similarity of toxic action falls on a continuum and the level of evidence required in the assessment. (It is recommended that users of this document coordinate such decisions with the relevant U.S. EPA programs, offices, or regions.) Low evidence of toxic similarity (e.g., simply the same target organ or similar chemical structures) could potentially be adequate for screening assessments or applications of the hazard index (HI), for example. Conversely, more detailed evidence of the same or similar toxicodynamics would be ideal for establishment of relative potency factors (RPFs).[31] Evidentiary bases between these two ends of the data-dependent continuum can be envisioned and whether such data suggest the chemicals comprise a single dose-additive group, multiple

[31]As stated in Section 2, some measure of relative potency is required to implement an RPF approach.

independent groups, or something more complicated, will require an assessment of the available toxicodynamic information and likely some scientific judgment. Confidence in the grouping decision and the resulting outcomes of the dose-additive models will depend on both the quality of evidence and the level of biological organization at which toxicological similarity is observed. To the extent possible, analysts should qualitatively characterize their overall confidence that the chemicals comprising the dose-additive group share a common toxicodynamic pathway or KE.

3.3.1. Types of Toxicodynamic Information

Different types of toxicodynamic information can be used to evaluate the degree of toxicological similarity. Toxicity information could include any of the following: structure-activity analyses, in vitro studies, (ex vivo) cellular or tissue studies, and in vivo whole-animal studies, as well as dose-response analyses and toxicodynamic models based on those types of data. The studies could encompass both traditional toxicology studies (e.g., 90-day test animal bioassays) and NAMs such as those described in *Toxicity Testing in the 21st Century* (NRC 2007). For environmental mixtures, the quality, quantity, and relevance of the evidence that the chemicals specifically share a toxicodynamic pathway or share a KE could vary across the chemicals. Lambert and Lipscomb (2007) provided an illustration of these concepts. When toxicodynamic models are available, the information can become more complicated. For some endpoints such as altered hormone synthesis, even simple models involving competitive antagonism based on equilibrium binding and mass balance can suggest joint toxicity that is greater than, consistent with, or less than concentration addition (Webster 2013). How to decide toxicological similarity from such a diverse array of toxicodynamic evidence is still an evolving field of study.

3.3.2. Empirical Evidence of Dose Addition

Empirical evidence from mixture studies[32] that is consistent with dose-additive component-based estimations of mixture toxicity could provide significant support for a decision to group some chemicals under an assumption of dose addition, particularly when the other evidence is weaker (U.S. EPA 2000c). The converse is also applicable; that is, empirical evidence that mixture toxicity estimates derived from a whole mixture or from the integration of information from individual components, based on an assumption of dose addition, are inconsistent could decrease support to group chemicals. While empirical chemical mixture evidence is not essential to invoking an assumption of dose additivity, having such study data can increase confidence in the decision whether or not to group chemicals together, as well as

[32]This document assumes that generally there are no directly relevant, empirical toxicity data on the whole mixture or fractions of the mixture.

help determine whether deviations from dose addition are expected for certain conditions (e.g., at higher doses). Section 4 contains further discussion of approaches and concepts involved with evaluation of mixture and chemical component data for consistency with dose addition. It is incumbent upon the risk assessment practitioner to present and characterize their confidence as to whether the similarity evidence used to develop dose-additive groups for an assessment was sufficiently strong and whether the uncertainties were adequately characterized (see Section 3.3).

3.4. COMPARISON OF MIXTURE ADDITION METHODS

This section illustrates the underlying toxicological pathway information for three different, component-based mixture methods (see Section 2 for details): dose addition (described using the RPF method), response addition, and integrated addition.

Figure 3-5 illustrates the underlying toxicodynamic information for three different hypothetical mixtures of environmental chemicals. Differences in the toxicodynamic information indicate support for selecting from among one of the following three different component-based mixture methods: dose addition (left column), response addition (center column), and integrated addition (right column), which combines both dose and response addition (see related discussions in Sections 3.2, 3.3, 3.5, and 3.6, and additional types of data that are becoming increasingly available to inform such decisions in Section 5). The formulas for calculating the total mixture risk (i.e., probability) of a common effect (r_{mix}) are shown for each method. The key dose-additive events are indicated by the stars within the rectangles. Additional intermediate events or processes before or after the key dose-additive event are indicated by the ellipses within the ovals.

In Figure 3-5, when applying dose addition using the RPF method (left-hand column of Figure 3-5), the chemicals in the mixture are assumed to cause a specific adverse effect by a common MOA. This method requires identification of an index chemical that has both exposure and dose-response information. For each mixture component chemical, this method requires exposure information (e.g., oral route in mg/kg-day) and sufficient hazard and dose-response information to estimate the potency of each chemical relative to the selected index chemical. An index chemical-equivalent dose (ICED) can be estimated for each component as the product of the corresponding RPF and exposure, and then summed for the ICED of the mixture, $ICED_{MIX}$. The $ICED_{MIX}$ is then mapped to the dose-response function for the index chemical (see discussion in Section 2.2.2 and Eq 2-17) to estimate the response associated with the mixture of component chemicals. Note that for the RPF approach, a hazard quotient (HQ) or margin of exposure for the mixture could also be estimated (not shown in Figure 3-5).

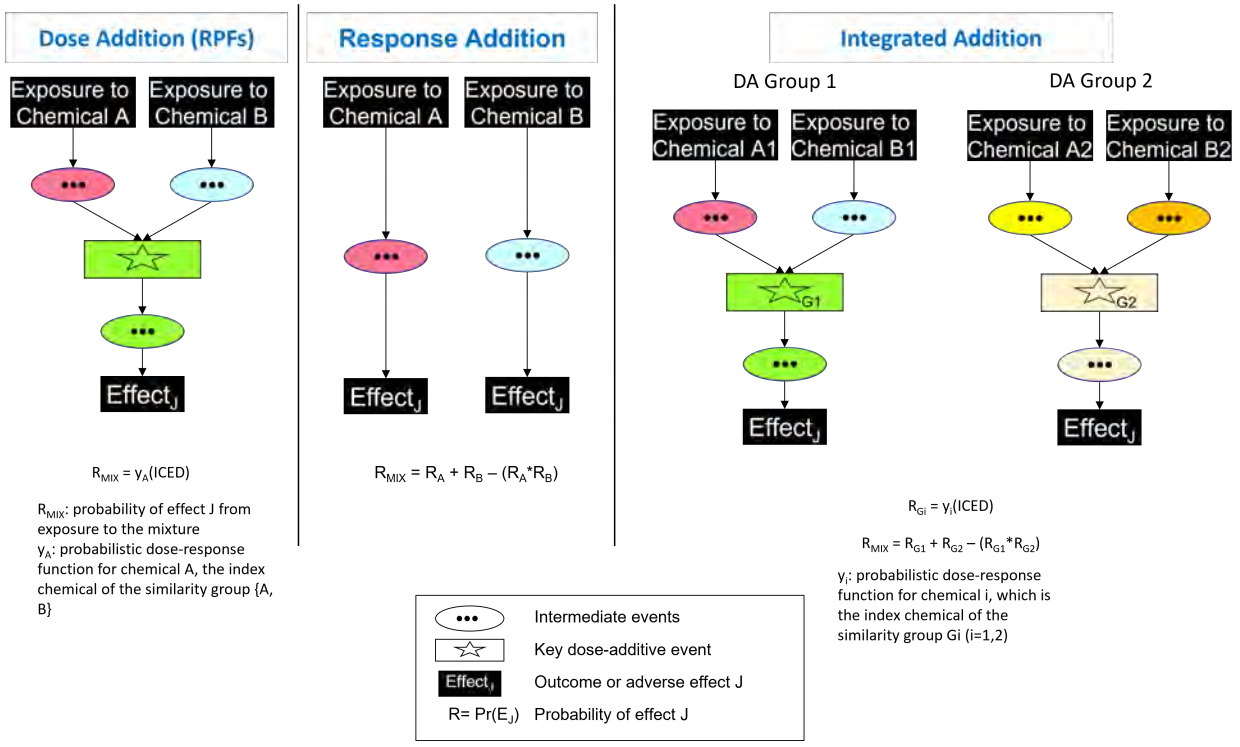


Figure 3-5. The information on mechanism/mode of action influences the choice of component-based risk formula for chemical mixtures.

Note: While dose addition can be used to estimate measured responses, here we restrict the response function *y* to be the probabilistic risk function for the specific effect J. For integrated addition, the analyst follows dose addition for each similarity group (G1 or G2) to estimate each group's risk, and then uses response addition to combine the two group risks. Groups G1 and G2 likely have different dose-additive KEs.
ICED = index chemical-equivalent dose; KE = key event; RPF = relative potency factor.

When applying response addition (center column Figure 3-5), the chemicals in the mixture are assumed to cause their toxicity through independent toxicodynamic pathways (see Sections 2.1.4, 2.1.5, and 3.2 for discussions of independence). For each component, this method requires exposure information and a dose-response function, as well as the assumption of no toxicokinetic interactions between chemicals/pathways. The single chemical risks can be separately estimated and then summed as shown by the equation in the center column to estimate the mixture risk (see discussion in Section 2.1.4 and Eq 2-3).

Components of some mixtures can affect the same health outcome through a network of same/similar, convergent, and different MOAs. Integrated addition, which is depicted in the right-hand column of Figure 3-5, may be appropriate for estimating risks from mixtures that act through network variations along source-to-outcome continuums that ultimately induce the same health effect. This method requires data-driven assignment of "like" mixture components to MOA groupings and identification of an index chemical for each operant MOA ("operant" meaning that there is evidence that a given MOA is related to the given health outcome of

concern). All components in a specific MOA grouping are assumed to be dose-additive. For each component chemical member, this method requires exposure information and sufficient dose-response information to estimate the potency of the chemical relative to the index chemical for the MOA grouping. An ICED can be estimated for the components comprising each MOA grouping, and the MOA-specific group response can be calculated using the dose-response function for the appropriate index chemical (see discussion in Section 2.2.4). Then, using response addition, the predicted risk for the whole chemical mixture is estimated by summing the predicted risks across the MOA groupings using the formula shown in the right-hand column of Figure 3-5 (see also Figure 2-3).

3.5. DEVELOPING DOSE-ADDITIVE GROUPS IN PROGRAM OFFICE AND REGIONAL APPLICATIONS

Several U.S. EPA offices have advanced and implemented considerations for developing groups of dose-additive chemicals to fulfill their statutory obligations regarding mixture risk. Their practices and guidance were developed with program-specific regulatory and policy aspects in mind.

3.5.1. Hazard Index for Superfund Site Assessments

The HI was the first application by U.S. EPA of a dose-additive method to guide decisions at Superfund sites [U.S. EPA (1986); see also relevant discussions in Section 2]. A two-stage grouping approach is often used. The first stage typically includes all chemicals of potential concern that are identified at a site; that is, all chemicals are included in a single group. The hazard associated with this group is then evaluated using the HI_{ALL}. In this application of the HI, exposure estimates and a health reference value (RfV), irrespective of the critical effect on which each RfV derivation is based, are needed for each chemical included.

A second HI might be calculated if the HI_{ALL} exceeds 1 or otherwise indicates possible concern of mixture risk. This second HI uses chemicals grouped by target organ (i.e., HI_{TO}), thus producing a separate HI for each target organ of concern (see Section 2.2.1.1).

3.5.2. The Office of Pesticide Program's Process for Developing Dose-Additive Groups of Pesticides When Implementing the Food Quality Protection Act

U.S. EPA's Office of Pesticide Programs (OPP) has used the concept of similarity of toxic action to identify common mechanism groups (CMGs)[33] of pesticides for application of the RPF approach in implementing the Food Quality Protection Act (FQPA) (U.S. EPA 1999; 2002a) and the Federal Food, Drug, and Cosmetic Act (U.S. EPA 2016). The OPP approach consists of two stages: Stage 1, forming the candidate set of chemicals, and Stage 2, deciding on the final CMG, where the common mechanism has been interpreted as a shared MOA [i.e., the chemicals cause the same toxic effect by essentially the same sequence of KEs (U.S. EPA 2002a)]. Note that the candidate group can include parent chemicals and metabolites that are themselves members of the group. The main characteristics evaluated for the candidate group include one or more of the following (U.S. EPA 1999):

- Structural similarity
- Similarity of mechanism of pesticidal action
- Shared general mechanism of mammalian toxicity
- Shared toxic effect

The final CMG is determined by an extensive review of information on the toxic MOA for each of the candidate chemicals and is "conducted in a manner similar to that used by U.S. EPA in its pesticide registration and reregistration programs" (U.S. EPA 1999). The final criterion of sharing a common MOA typically includes a comparison of data on chemical structure, toxicokinetics, and toxicity. U.S. EPA (1999) noted that, "generally, the more that is understood about the various biochemical events that lead to a toxic effect, the more apparent and scientifically acceptable is the mechanism of toxicity."

The U.S. EPA (2016) Framework for Screening Analysis for Pesticides describes a two-step screening approach for cumulative risk assessments of pesticides. The approach begins with evaluation of available toxicological information; if needed, this is followed by a risk-based

[33]The 1996 FQPA uses the word "mechanism." OPP's guidance documents have consistently interpreted that statutory use of "mechanism" to be the same as MOA. The U.S. EPA (2016) Framework for Screening Analysis for Pesticides further explains that "This definition of mechanism of toxicity is similar to the concept of MOA." The U.S. EPA (1999) Common Mechanism Group guidance document for pesticides describes "mechanism of toxicity as the major steps leading to a toxic effect following interaction of a pesticide with biological targets." U.S. EPA (2016) further explains that "all steps leading to an effect do not need to be specifically understood. Rather, it is the identification of the crucial events following chemical interaction that are required in order to describe a mechanism of toxicity." It further explains that "This definition of mechanism of toxicity is similar to the concept of MOA as defined by U.S. EPA's Cancer Guidelines (U.S. EPA 2005a) and other international efforts through the -OECD and World Health Organization (WHO) (Boobis et al. 2008; Meek et al. 2014; Seed et al. 2005; Sonich-Mullin et al. 2001)."

screening approach. This effort is intended to supplement the OPP's existing guidance documents for establishing CMGs and conducting cumulative risk assessments.

3.5.3. Toxicity Equivalence Factors for Dioxins and Dioxin-Like Compounds

The first dose-additive risk prediction for a mixture was developed for exposure to dioxin and dioxin-like compounds (DLCs). The motivation was the universal co-occurrence of many DLCs along with minimal or nonexistent dose-response data on most of the congeners. The toxicity equivalency factor (TEF) formula allowed congeners with poor quality dose-response information to be assessed by scaling the dose-response data of a well-studied congener (see Section 2 for details). The basis for this "simple similar action" scaling approach and subsequent application of dose addition for the mixture was the scientific consensus that TCDD and DLCs acted toxicologically by a shared mechanism of action that was initiated by the binding of a dioxin or a DLC to the aryl hydrocarbon receptor (AhR). The TEF approach has been applied by U.S. EPA and the World Health Organization (WHO) to TCDD and DLCs, including polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and dioxin-like PCBs (Ahlborg et al. 1994; Barnes et al. 1991; U.S. EPA 1987; 1991a; 2008; 2010; Van den Berg et al. 1998; Van den Berg et al. 2006). The U.S. EPA TEF guidance also states that use of the TEF approach assumes that all cancer and noncancer health effects are AhR-dependent, so the TEFs apply to all apical effects mediated through the AhR and for all exposure routes and durations (U.S. EPA 2010).

U.S. EPA has suggested grouping characteristics for evaluating which compounds to include in the TEF approach for dioxins and DLCs (U.S. EPA 2010). The current steps for grouping dioxins and DLCs when applying the TEF approach were developed by the WHO (Van den Berg et al. 2006). The list of characteristics offered by Van den Berg et al. (2006) include the following:

- Structural similarity to PCDDs or PCDFs,
- Capacity to bind to the AhR,
- Capacity to elicit AhR-mediated biochemical and toxic responses, and
- Persistence and accumulation in the food chain.

U.S. EPA (2010) recognizes that the full body of evidence indicates that AhR "binding appears to be necessary—but not sufficient—to generate the wide variety of toxic effects caused by dioxin-like halogenated aromatic hydrocarbons." A lack of knowledge about AhR binding was not considered to be sufficient justification for excluding a chemical from the TEF group (U.S. EPA 2010).

3.6. UNCERTAINTIES WHEN DEVELOPING DOSE-ADDITIVE CHEMICAL GROUPINGS

When conducting mixture risk assessments, depending on the scope of the application and statutory requirements, it may be necessary to identify and characterize the uncertainties associated with the applied grouping considerations (e.g., Section 3.2). First, the potential for classification error needs to be considered. Following the similarity of toxic action considerations (see Section 3.2) from the top down (most to least relevant) is an increasingly *inclusive* classification process, but with increasing uncertainty in the relevance of (i.e., toxicological similarity of) the members included in the group ("inclusion uncertainty"). Conversely, it is an increasingly *exclusive* classification process from the bottom up, with uncertainty increasing as to the completeness of the membership in the group ("exclusion uncertainty"). Membership decisions for chemical groups based on any of the considerations will have elements of both uncertainties, but in different proportions depending on the strength of the evidence for similarity of toxic action. In summary, classification error may overestimate the total mixture dose (i.e., the total dose for the mixture dose-additive group under evaluation) when chemicals are included in a dose-additive grouping based on non-MOA data (e.g., structure; physicochemical properties), and will underestimate it when chemicals are mistakenly excluded from the group (e.g., different MIEs across component chemicals but pathways converge at a downstream intermediate KE).

For the less exclusive considerations (e.g., similar dose-response shapes, similar structure, or same target organ as discussed in Section 3.2), a primary concern is whether unrelated chemicals are included in the group, potentially leading to an overestimation of total mixture dose (or mixture equivalent dose), generally resulting in an overestimation of hazard or risk.[34] That overestimation of hazard/risk will occur if the (overestimated) mixture dose is "plugged in" to an existing dose-response function (e.g., of an index chemical). As an illustration, consider Figure 3-2, where Chemicals A, B, and C are essentially "toxicodynamic clones," exhibiting the same MIEs and affecting the same toxicodynamic pathways. If one of these three chemicals were not considered to be a member of a dose-additive group, the total mixture dose would be underestimated. Less commonly, the combined component data may be used to develop a multivariate response surface predictive model. In this case, an overestimation

[34]The overestimation of mixture hazard/risk will occur if the (overestimated) mixture dose is "plugged in" to an existing dose-response function (e.g., of an index chemical). Less commonly, the combined component data may be used to develop a multivariate response surface predictive model. In this case, an overestimation of the dose (by including non-similar chemicals) will lead to an underestimation of the dose coefficients for the truly similar chemicals. When applied to a mixture with a smaller fraction of that unrelated chemical, the model will then underestimate the mixture response. If applied to a mixture with greatly increased fraction of the unrelated chemical. the error could be the reverse, an overestimate of the true mixture response. See Section 4 for a discussion of combined-data modeling for estimating a mixture dose-response function.

of the dose will lead to an underestimation of the dose coefficients for the truly similar chemicals. When applied to a mixture with a smaller fraction of that unrelated chemical, the model will then underestimate the mixture response. If applied to a mixture with a greatly increased fraction of the unrelated chemical, the error could be the reverse and overestimate the true mixture response.

These uncertainties need to be characterized qualitatively both for the chemicals individually and the grouping(s) collectively, regardless of which dose-additive method is being used; these uncertainties also need to be placed into the context of the purpose of the mixture risk assessment. The uncertainty characterizations will reflect the kind of information available, its scientific quality, and its relevance for evaluating group membership; the information will likely vary in both quantity and quality among individual chemicals comprising the group. For example, group membership for some mixture components may be completely dependent on some amalgamation of nonapical effect data (e.g., in vitro cell-based bioactivity NAMs, toxicogenomics, structure-activity relationships/read-across, etc.). It is critical to leverage such data sources, as health outcome data are available for only a small fraction of the thousands of legacy and emerging chemicals in commerce and the environment. It is important to note that gradations of uncertainty will likely be a persistent challenge for inclusion/exclusion decisions associated with group membership due to inherent diversity in the types and quantity of available hazard and dose-response data across mixture component chemicals. When the variability of the information available across all the chemicals of a group is large, a characterization of the uncertainty associated with the major contributors to any estimate of mixture risk can be insightful. In addition, for assessments with important ramifications or for those assessments addressing statutory requirements, an analysis of uncertainty may be important.

Sensitivity analyses based on alternative groupings can be performed to characterize the quantitative impact of potential differences in membership in a dose-additive group. A sensitivity analysis could be performed by constructing alternate HIs, taking into consideration such factors as the range of possible HQ values for specific chemicals characterized as highly uncertain. A range of HQ values could be bounded by exclusion of chemicals with weaker evidence in support of toxicological similarity and inclusion of chemicals marginally excluded from the group. The sensitivity analysis could be a valuable tool for quantitatively evaluating the impacts of the chemical grouping in the subsequent mixture risk assessment.

4. ADVANCES IN QUANTIFYING RELATIVE POTENCY FOR COMPONENT-BASED ADDITIVITY METHODS

This section discusses advances in methods for predicting chemical mixture risk when dose-response data are available for the mixture chemical components, but there is insufficient information on the whole mixture to develop a dose-response model from the mixture itself. Additionally, the focus is on advanced quantitative predictions, rather than the hazard index (HI), but still using only individual component dose-response data. One goal of this chapter is to review dose-addition methods (published since 2000) that address limitations in the HI, relative potency factor (RPF) and other U.S. EPA mixture methods that are based on the Finney definition of dose addition, particularly limitations from the assumption of constant relative potency across the dose range of application. The focus is on predictive methods, because there generally will be little opportunity to evaluate predictions at the low(er) end of environmental exposure ranges that are typically of most concern for humans. Experimental rodent bioassays often lack the ability to characterize clearly the responses in low exposure ranges as the dose or concentration levels are typically selected to ensure higher probability of toxic response, and there is rarely enough information from epidemiological studies to characterize dose-response in a typical environmental exposure range.[35] Thus, the focus here is on the conceptual and theoretical basis for developing dose-response functions in the observed range for animal bioassays before considering extrapolation to likely lower human environmental exposures. Throughout this section, it is assumed there are sufficient data to develop dose-response functions or RPFs for all of the mixture components (Section 2.2.2 and Table 2-3 address RPFs). Note that for simplicity, the toxic health outcome in any specific assessment is assumed here to be similar for all mixture components. Not included in this report is the development of complete mixture dose-response functions to simultaneously address a suite of different endpoints.[36] A primary theme throughout this section is that consideration of dose-additive methods should not be dismissed too early and might be considered throughout the assessment process. At least two scenarios that might have been considered as justification for not using a dose-additive risk assessment method in the past are described. These scenarios, which include different component dose-response curve (DRC) shapes and different molecular initiating events (MIEs), are described in detail below.

This section begins with an overview of generally applicable dose-additive methods for chemical dose-response data and follows with a discussion of specific situations that require

[35]The topic of low-dose extrapolation is further discussed in Sections 4.1.3.2 and 4.1.4.

[36]One exception is a suite of related effects arising directly from the same toxicodynamic pathway, of which the appearance of any one of them at the same exposure level would constitute a positive response (see discussion and examples in Section 3.2).

modifications to, or limitations of, the general case. For the general case, options for dose-additive mixture models using explicit RPFs and those not using RPFs are described (see Section 4.1). A more detailed discussion of U.S. EPA's standard method, the index chemical, and RPF model (IC/RPF model), is presented in Section 4.1.1 (see also Section 2.2.2), along with qualitative considerations for deriving RPFs (see Section 4.1.2). Modifications to the standard U.S. EPA approach for the specific case of different chemical component DRC shapes are also discussed in detail (see Section 4.1.3). Issues concerning the application of dose-additive models to continuous measures are then presented, along with possible modeling solutions.

Overall, there are two general issues that apply to both dichotomous and continuous-measure health outcomes and two additional issues that apply primarily to continuous outcomes. The first general issue is different DRC shapes among the chemical components. This is an issue because of U.S. EPA's use of the Finney definition of dose additivity, where components behave toxicologically as if they were dilutions of each other and therefore should have similarly shaped DRCs. That leads to the requirement, when using the RPF method, for relative potencies (RePs) to be constant across the dose range of interest ("constant relative potency"); different DRC shapes lead to dose-dependent relative potency.

The application of dose addition to a mixture implies that joint toxicity is dependent upon a key step somewhere along the modes of action (MOAs) or adverse outcome pathways (AOPs) of the components, spanning the continuum from external exposure to adverse outcomes (AOs). If the DRCs are similar and are based on external exposure levels, then another assumption is that the relevant toxicokinetic and toxicodynamic processes prior to the key event (KE), when the dose addition occurs, do not differ much across the components. When otherwise similar components show different curve shapes, one interpretation could be that the toxicokinetic and toxicodynamic processes are not proportional across the components and also may be dose-dependent in a manner that differs across the components.

These ideas about different DRCs are important because there is a common assumption in the literature and in practice that only two options can be used for predicting the mixture response: dose (concentration) addition and response addition (independent action). That misconception unfortunately has resulted in the interpretation of differing component DRC shapes as evidence for independence of toxic action and the need to apply response addition, rather than dose addition. However, as will be discussed in Section 4.1.3, this is not necessarily the case. Furthermore, whether the DRC shapes in the observed region are the same or different, U.S. EPA emphasizes that there is no expectation that the shape will be the same below the observed dose region. Section 4.1.3 provides a further discussion of dissimilar DRCs and presents methods applicable to the situation, including ideas for modeling in the low-dose/low-response region. One caution is that if the prediction of the mixture response uses

external dose, then differences in DRCs will indicate an inaccurate mixture prediction based on dose addition. While downstream dose addition can argue against use of independent action approaches and will advance understanding of joint toxicity, it will have no practical impact until tissue doses can be feasibly measured or estimated to use instead of external doses.

The second general issue is the finding that there may be differences in the initial interactions of the chemical components with the biological system. Specifically, a finding that the MIE is not the same for all mixture components might be construed as evidence for addressing those different components using methods other than dose addition. However, if the toxicodynamic pathways converge at a common dose-additive process downstream from the MIE (as with Chemicals D and E in Figure 3-4), dose-addition models may still apply. Section 4.1.4 provides a further discussion of this issue.

Two other issues that apply only to continuous measures, such as serum enzyme activity or hormone levels, are discussed in Section 4.2. The first of these issues is a difference in control values (mean or variance) among the component studies. If the toxicological metric of interest is the absolute value of the measure (i.e., not scaled to the control mean), a difference in control means or control variances will result in a mixture risk prediction that is dependent on the choice of the index chemical in the RPF model (Chen et al. 2003). The second continuous endpoint issue is partial agonism, in which the components do not elicit the same maximal response. The presence of partial agonists in the mixture makes it difficult to apply dose addition to estimate mixture risk above the saturating dose of the lowest partial agonist maximum response. Scaling the responses to the range of the full agonist may not resolve this issue if the absolute value of the measure is biologically important. Sections 4.1.3.2 and 4.2 include further discussion of this issue and a description of some published methods that address partial agonism.

4.1. GENERALLY APPLICABLE DOSE-ADDITION METHODS FOR PREDICTING MIXTURE RISK

This section discusses dose-addition methods for risk prediction when the AO is expressed either as the fraction of the population (dose group) exhibiting a specific effect or as the mean of a continuous measure for a dose group. The following discussion assumes that dose-response information is available for all mixture components, either in the form of functional dose-response model fits or relative potency estimates. As mentioned in the introduction to this section, the primary issues in the choice of method are the degree of independence of toxic action (e.g., lack of toxicological similarity in MOA/AOPs) and similarity of DRC shape. The first step in establishing the appropriate method is to determine whether the mixture components share a similar toxic action, act by independent pathways, or have a more complex joint action in eliciting a specific effect. While U.S. EPA guidance (U.S. EPA 2000c)

allows for consideration of independence of toxic action among component chemicals (i.e., response addition), specific guidance is lacking on how to determine such independence. Unless there is evidence that there are no common KEs or processes in the respective toxicity pathways (TPs) for chemicals affecting the same target organs, similarity of toxic action and dose additivity is usually assumed as a default at U.S. EPA, with modifications allowed to address known toxicological interactions (U.S. EPA 2000c).

Next, the component DRC shapes are evaluated to determine whether constant relative potency might hold in the dose range of interest (the range of the intended mixture risk prediction) (see Section A.1 for considerations when testing the similarity of component DRC shapes). The decision of whether DRC shapes are geometrically similar affects the assumption of constant relative potency among the individual mixture chemical components and informs the choice of approaches for estimating relative potency. A lack of constant relative potency has often been interpreted as sufficiently weak evidence of similarity so that dose addition would not apply. Because of the frequent assumption (stated above) that the only alternative is independence of toxic action, nonconstant relative potency has been construed as a reason for preferentially selecting response addition over dose addition, but this is not necessarily justified. DRC shape is usually determined from the full range of experimental doses and can be subject to high-dose influence (see Section 2.1.6) that could result in different curve shapes. Constant relative potency may still apply downstream of the effective internal concentration delivered to the key dose-additive process. Section 4.1.3 discusses conditions for which dose addition would still be preferred for dissimilar curve shapes. Section 4.1.4 discusses the use of dose addition for the "convergent pathway" scenario previously described (see Section 3.2 and the MOA for Chemicals D and E in Figure 3-4), in which the initial chemical/tissue interaction processes are independent but in which the toxicodynamic pathways converge in a common process that leads directly to the AO.

4.1.1. Methods Based on Relative Potency for Similarly Shaped Dose-Response Curves

The concept of similarity in dose-response in environmental chemical mixtures risk assessment, and its practical applications, has been a subject of debate in the literature. The characteristics used to describe similarity of DRCs across the component chemicals of a mixture range from strictly (log-dose) parallel lines (Finney 1971) to similarly shaped curves over the dose range of interest [as assumed in U.S. EPA (2000c)], to no assumption at all regarding shape (e.g. Berenbaum 1985). The theoretical support for dose addition described in the 1986 Chemical Mixtures Guidance (U.S. EPA 1986) and 2000 Supplementary Chemical Mixtures Guidance (U.S. EPA 2000c) is based on toxicological similarity, such that similar chemicals may be substituted at a constant proportion for each other [U.S. EPA (1986; 2000c), citing Finney

(1971)]. Bliss (1939) described this theoretical basis and defined chemical dose additivity as a consequence of a "similar joint action," such that the chemicals behave as dilutions of one another.[37] This property implies that doses resulting in the same magnitude of response will be proportional across the response range of concern ("constant proportionality"),[38] which in turn implies that the shapes of the DRCs will be the same over this range.[39] In terms of dose-response modeling, the same DRC-shaped property means that the same mathematical dose-response function, except for the dose coefficients, applies to all components and to the mixture itself (Berenbaum 1985; Hertzberg et al. 2013; Meadows et al. 2002). However, a finding of different shapes for DRCs does not mean that dose additivity does not hold, as is discussed in the next section. Conversely, if the component DRC shapes are the same, dose additivity does not necessarily hold: complex interactions could be operating [see binary mixture example in Hertzberg et al. (2013)] or independence of toxic action might apply.

In the Agency-wide guidance on dose addition, there is an assumption of constant relative potency (U.S. EPA 1987; 2000c), but a demonstration of empirical evidence, such as similar DRC shapes, is not required. While the "Bliss dilution" concept was used by U.S. EPA as the operational definition of dose addition, it was not intended as a rigid and absolute requirement for dose-additive risk estimation, nor as a strict representation of the physical and biological behavior of similarly acting chemicals (U.S. EPA 2000c). Specifically, the same mechanism of action is not required for dose addition to hold. In the case of TPs merging at a common key toxicodynamic event, U.S. EPA generally assumes standard dose-addition methods are applicable for estimating the risk posed by the mixture, provided the DRC slopes are similar. The next two sections discuss the biology of toxicological similarity in greater detail and describe procedures for approaching mixture risk assessment when the components have different dose-response shapes.

4.1.1.1. Relative Potency Factor Approach

As stated in Section 2, U.S. EPA has long used RPFs for computing equivalent doses, e.g., for use in determining pesticide margins of exposure or cancer risk estimates for poorly studied chemicals and for mixture response prediction when toxicological similarity and constant

[37]Loewe and Muischnek (1926) may have been the first to describe this definition of dose additivity, often termed "Loewe additivity," but without Bliss's intuitive dilution analogy.

[38]Constant proportionality, synonymous with constant relative potency, refers to the proportionality of doses associated with a specific effect level for all exposure levels (U.S. EPA 2000b). That means the effective dose (ED_x) ratios are the same for all x: $ED_{10}A/ED_{10}B = ED_{20}A/ED_{20}B$ and so on.

[39]In the restricted sense, for functions that have shape parameters (e.g., Weibull power or Hill coefficient), "same shape" means that the curves follow the same functional form with the same shape parameter. The curves can differ in the "location" parameter (e.g., ED_{50}). In the general sense, the -DRCs are geometrically similar and will look parallel when the response is plotted against the logarithm of the dose.

relative potency can be assumed. In the RPF method for predicting mixture response, an index chemical is chosen from among the mixture components, and its dose-response function serves as the predictor of response to the mixture when all other chemical component doses are scaled to the index chemical by means of RPFs. RPFs are computed as the ratio of doses eliciting the same effect magnitude, with the index chemical dose in the numerator, as in Eq 4-1 (a generalization of Eq 2-15).

$$RPF_i = \frac{ED_{X_{IC}}}{ED_{X_i}} \tag{4-1}$$

where:

RPF_i = the RPF for chemical *i*,

$ED_{X_{IC}}$ = the index chemical dose eliciting the specific effect level *x*, and

ED_{X_i} = the dose for chemical *i* eliciting the specific effect level *x*.

The effect level *x* is generally a percentage value, reflecting either the proportion of a population responding to a dichotomous outcome or the percentage difference from a control mean value for a continuous measure. For continuous endpoints, *x* could also be expressed as one standard deviation from the control mean or a fixed mean response value. ED_x values can be derived from functional dose-response model fits or simply by observation of the data.

Whatever the assigned value, for RPF calculations *x* must be the same for all mixture components. In the latter respect, the use of RPFs assumes constant relative potency [termed "constant proportionality" in U.S. EPA (2000c)]. In the strictest sense, constant relative potency means that the RPF will be the same for any response level, be it 1%, 10%, 50%, or 99%. This property holds only if DRCs have the same geometrical shape, e.g., as shown by a parallel appearance on a semilogarithmic plot (i.e., log-dose). In a practical sense, however, constant relative potency only needs to remain below the response level at which the RPF was computed, that is, within the low-dose region for prediction of mixture risk. If the dose-response shapes are not the same, dose-addition methods can still be used, but with some modification and additional assumptions as discussed in Section 4.1.2.

If adequate dose-response functions are available for all mixture components, and the DRC shapes are similar, the basic RPF dose-addition model can be used effectively. Examples of methods for determining similarity of shape and computing RPFs from fitted parameter values for the models in U.S. EPA's Benchmark Dose Software (BMDS) are given in Appendix A. The RPF model is given in Eq 4-2 as a generalization of the dose addition example of Eq 2-2. Eq 4-2 is a functional form of Eq 2-19 where the summation in Eq 4-2 is the index chemical-equivalent

dose (ICED) of the mixture, defined in Eq 2-18. The predicted mixture response is then determined from the dose-response function of the index chemical.

$$\hat{y}_{\text{mix}}(d_1,\cdots,d_n) = f_{IC}\left(\sum_{i=1}^{n}(d_i\text{RPF}_i),\theta\right) \quad (4\text{-}2)$$

where:

$\hat{y}_{mix}$ = the estimated response to the mixture given component doses $d_1,\cdots,d_n$, and

f_{IC} = the index chemical dose-response function computed from the sum of the RPF-scaled component doses ($d_i\text{RPF}_i$), with parameter vector θ.

The RPF_i for the index chemical is given the value of 1 (i.e., the numerator and denominator in Eq 4-1 are the same value), with all other RPF_i values calculated relative to the potency of the index chemical. As noted in Section 2.2.2, the index chemical is usually the chemical with the most extensive toxicological database, for a given exposure route, duration, health effect domain, and/or dose range in the group or mixture being assessed. The dose-additive estimate of the mixture dose-response function is defined by the index chemical dose-response function. As a result, defining the dose-response model and its parameters for the index chemical is more important than developing models for the other components. In fact, in the early U.S. EPA application to mixtures of dibenzodioxins and dibenzofurans (U.S. EPA 1989a), estimates of RePs for several component were based not on dose-response models but on potency ratios of other biological measures. The response to the mixture can be estimated by substituting into Eq 4-2 the RPFs computed from the individual component dose-response functions, as in Eq 4-1. When dose-response data are available on all components, the RPFs can be estimated directly as parameters in the prediction model fitted to the merged data from all components. An example of the latter approach can be found in Chen et al. (2001).

4.1.1.2. Additivity Surface Approaches

Alternatively, a linear or nonlinear regression model can be used to estimate mixture risk from the combined data directly, without specifying an index chemical (e.g. Gennings et al. 2002; Hertzberg et al. 2013). All the model parameters are fitted simultaneously using the combined component data, with an assumption (following the Finney definition of dose addition used by U.S. EPA) that, except for the dose coefficients, the model parameters and curve shape are the same for all components and the mixture itself. An example of such an additivity surface model using the combined component data is depicted in Eq 4-3.

$$\hat{y}_{mix}(d_1,\cdots,d_n) = \alpha + \exp(\beta_0 + \beta_1 d_1 + \beta_2 d_2 + \ldots + \beta_n d_n) \quad (4\text{-}3)$$

where:

$\hat{y}_{mix}$ = the estimated response to the mixture given component doses $d_1, \cdots, d_n$,

$\alpha + \exp(\beta_0)$ = the background response term, and

β_i = the dose coefficients, for I > 0.

In additivity surface models such as Eq 4-3, no single chemical is given elevated importance (i.e., no index chemical); all components are treated as equal. With the Finney definition, the assumption is that, except for dose coefficients, all other model parameters are common across components. If the component model includes a shape parameter such as an exponent of dose (the example does not), then the individual component models could have component-specific shape parameters. The additivity surface model does not allow that, so it includes a single shape parameter that is a "blend" of the component dose-response shape parameters. For example, in Olmstead and LeBlanc (2005) the power parameter (their "slope of the curve") in the component models is replaced by an average power, such as the mean or median of the component power parameters. Additionally, RPFs are not explicit, but can still be estimated from ratios of the fitted dose coefficients. The main advantage of a combined component-data regression model (e.g., Eq 4-3) lies in not having to choose an index chemical; the mixture dose-response function is fitted directly from all the chemical component data. That property might also encourage more use of data specific to the mixture being assessed, instead of relying on a predetermined set of RPFs that might have been developed for a different scenario. However, in terms of predicting the most accurate mixture dose-response function for general use in risk assessment, this advantage is also the main liability. The primary reason for selecting an index chemical is that the best and most reliable data set can be used for the prediction, reducing the influence of potentially much lower quality data. In the same way, if RPFs are obtained from an additivity surface model, without an index chemical anchor, their values could be similarly influenced.

4.1.2. Qualitative Considerations for Determining Application of Relative Potency Factor Models

The ideal qualitative scenario for evaluating the consistency of information related to confidence in determining constant relative potency (similarity of dose-response shape) across a set of mixture components would be a data set for which all the following are the same:

- Toxicokinetics,
- AOPs (to a specific common endpoint),
- Test animals (species, strain, sex, age, life stage, source),
- Exposure protocol (route, vehicle, frequency, duration),

- Experimental design in addition to previous items (e.g., low-to-high-dose range, housing, feed, water, light-dark cycle, sample size),
- Laboratory,
- Investigators, and
- Temporal proximity (e.g., simultaneously conducted studies).

The consistency of the laboratory and investigators as well as the temporal proximity of experiments address concerns about consistency (i.e., repeatability) across studies (or a group of studies) that could be used to evaluate the relative potency of a group of chemicals. Unlike the other attributes (i.e., toxicokinetics, AOPs, test animals, exposure protocol, and experimental design), it is typically not feasible to control these attributes across experiments.

Furthermore, tests with all these attributes ideally would be available for multiple species. As mentioned previously, similarity of toxicokinetics among mixture components is a prerequisite for constant proportionality to hold for interpretation of mixture dose-response based on external exposures. However, even if differences in toxicokinetics result in nonproportional delivery of the component chemicals to the target tissue, dose addition may still be relevant for the delivered internal concentrations. Thus, detailed knowledge of a common MOA would be the ideal situation for establishing high confidence that the chemicals were acting in a dose-additive manner, although U.S. EPA recognizes that MOA information could exist along a continuum. In U.S. EPA mixture risk assessment theory and practice, additivity or deviations from additivity are evaluated using endpoints generally at the apical (phenotypic) level of organization and do not necessarily represent the process in the TP at which dose additivity occurs. However, if there are no dose-dependent processes on the path from the key dose-additive event to the apical endpoint, the shapes of the dose-response functions will not be affected. Otherwise, target-tissue exposure measurements are highly desired and could be definitive in establishing dose addition. In addition, a large number of exposure levels covering the full range of response and a large number of animals per dose group would be desired. Obviously, these attributes (in the bulleted list above) are largely shared by all component chemicals tested in a single study but rarely would be similar across studies. Commendable examples of the latter are the National Toxicology Program (NTP) studies on 2,3,7,8-tetrachlordibenzodioxin (TCDD) (NTP 2006b), the dioxin-like compounds (DLCs)—2,3,4,7,8-pentachlordibenzofuran (NTP 2006a) and 3,3′,4,4′,5-pentachlorobiphenyl (NTP 2006c)—and their mixture (NTP 2006d), in which most of these attributes were met. There are no studies evaluating the effect of deviations from these attributes on the determination of constant relative potency across chemicals, and it is unclear whether similarity of shape would hold in the absence of these attributes. No strong conclusions can be made from analyses of disparate experimental designs. However, dose additivity may still

be supported beyond the simple default assumption, and RPFs may still be derived, depending on the strength of the data. No clear advice can be given at this time on how to evaluate the strength of the data, but a continuum of evidence can be envisioned (see Section 3.2). As an example, if there is information for a specific tissue-level endpoint for the same species and exposure duration, but from different laboratories, greater confidence can be held for RPFs developed from those data than if the information were from different species and different exposure durations. Otherwise, RPF equivalence across data attributes remains an assumption. What might constitute a minimum data set for RPF development is still a case-by-case judgment call. The usefulness of a qualitative descriptor of the confidence in the data used to develop RPF estimates is increased the more it characterizes the data attributes listed at the beginning of this section.

Furthermore, U.S. EPA (2000c) highlighted the importance of including a qualitative characterization of the strength and relevance of the component toxicity data used to estimate each component's RPF and suggested that it be included in the overall characterization of risk. For example, consider a two-component mixture composed of Chemicals X and Y, for which the strength and relevance of the toxicity data underlying the RPF estimate for Chemical X is judged to be "low" and the strength and relevance of the toxicity data underlying the RPF estimate for Chemical Y is judged to be "high." If the risk estimate for the mixture was based primarily on the chemical with the highest ICED, e.g., Chemical X, the assessment could be qualified in the following manner: "Most of the index chemical-equivalent dose comes from the contributions of Chemical X, for which the RPF is judged to be of much lower quality than the minor component, Chemical Y."

4.1.3. Dose-Addition Methods for Dissimilar Dose-Response Curves

If the DRC shapes are not the same across mixture components[40] but the components are otherwise known or assumed to act via the same MOA, the relative potency estimate will vary with the response percentage (the x in ED_x) at which it is calculated (Cornfield 1964; DeVito et al. 2000; Dinse and Umbach 2011; Hewlett and Plackett 1952; Meier et al. 1993; Thompson 1948). Some approaches to mixture dose-response assessment have only considered dose addition versus response addition (similarity versus independent action). Without considering the similarity of toxic action first, different component dose-response shapes could be mistakenly interpreted as evidence of different MOAs and as suggesting application of response addition (Dinse and Umbach 2011). However, dose (as delivered concentration) addition can still apply at the target tissue.[41] For such a situation, when using an RPF approach without incorporating

[40]For example, if the model equation has exponents on dose then those exponents will not be the same and the curves will not be parallel when response is plotted against the logarithm of dose.
[41]That is, the dose addition will apply to the internal dose at the MIE, rather than to the portal-of-entry exposure.

knowledge of the effective target-tissue dose, the predicted mixture risk computed from the exposures will depend on the choice of index chemical.

The issue of the dependence of mixture risk prediction using RPFs on the choice of index chemical was first shown by Chen et al. (2001) and was presented in U.S. EPA (2003b), but without much discussion. If there is a difference in the dose-response shapes of the mixture components, the predicted mixture risk will not be the same for each component chosen as the index chemical. Bosgra et al. (2009) provided the most succinct explanation of this phenomenon. They argued that for a mixture of iso-EDs (e.g., same individual ED_x) for two chemicals with differently shaped DRCs, the sum of the doses in either function chosen as the index chemical function would be $2 \times ED_x$ for that function. Further, because the putative index chemical functions have different shapes, the responses rise at different rates with increasing dose, therefore, the response at $2 \times ED_x$ for one function would not be the same as that for the other function. The underlying issue is prediction of the shape of the mixture DRC, which might not be equal to any one of the individual component shapes. Both Chen et al. (2001) and Bosgra et al. (2009) recommend that, when using an index chemical RPF approach with components exhibiting different DRCs, the results include a range of mixture response estimates to reflect the different choices of index chemical.

If the component DRC shapes are different, other approaches are available. However, these approaches require more data than a simple RPF approach does; full DRCs generally are required for each of the mixture components to implement most of the applicable methods. Any approach not based on dose addition must be clearly described, particularly the conceptual or empirical evidence supporting the alternative approach. In particular, "the evidence for applicability at low doses must be presented" (U.S. EPA 2000c). The RPF approach can still be used with different DRC shapes, but some assumptions about dose-response behavior in the low-dose and high-dose ranges and a modified RPF computation are needed. For example, if evidence of partial agonism (differences in maximal response) exists with some components, then the application of RPFs or any constant relative potency approach can be restricted to responses less than the smallest maximum component response. Alternatively, the dose addition model can be modified to address the partial agonism conditions, as discussed in Section 4.1.3.2.

Several alternative approaches are available from the literature or from U.S. EPA practices for predicting mixture risk for dose-additive chemicals with different DRC shapes. These approaches include:

- Focus on the low-response range with restricted application to assessments involving low doses or low responses by computation of RPFs at low-response levels, assuming

that they will be constant at lower response levels (e.g., at the ED_{10}) or by dose-response modeling using only data in the low-response range;

- Use of interaction index methods, which allow different, component-specific dose-response models, e.g., estimation of the total mixture ED_x from a simple transformation of the interaction index formula into the harmonic mean formula;
- Development of biologically based dose-response models that reflect differences in DRC shapes across components.

4.1.3.1. Approaches for Low-Response Levels

The application of mixture risk assessments generally to low environmental doses or low responses (e.g., low response rates in experimental toxicological studies) can facilitate some dose-response assessments with certain assumptions that help simplify the approaches and avoid some of the difficulties previously mentioned (see Section 2.2.2). In the first such approach, RPFs are defined as ED_{xIC}/ED_{xi}, where x is the common response level, the subscript "IC" refers to the index chemical and the subscript i refers to the other mixture components. If all the mixture component DRC shapes are the same, RPFs will be the same at whatever response level they are computed. For same-shape curves, the location parameter (for fitted models containing such a parameter, often near the ED_{50}; see Table A-1 and Table A-2) is a convenient basis, because it can be used directly without the need to compute an ED_x. In addition, there is usually less uncertainty (i.e., a narrower statistical confidence interval) in the ED_{50} than in lower ED_x values. However, as discussed in the previous section, when component DRC shapes differ, the RPFs depend on the response level at which they are computed. Therefore, a common practice is to compute RPFs at lower response levels than the ED_{50}, such as the ED_{10} or ED_{05} (U.S. EPA 2000c; 2007a; 2016). The intent of this approach is to estimate RPFs closer to the low-dose range of interest and to reduce possible high-dose influence on the RPF. This method has been used to assess pesticide mixture risks by U.S. EPA Office of Pesticide Programs (OPP) (U.S. EPA 2016) and is described in the literature (Budinsky et al. 2006; Toyoshiba et al. 2004). An implicit assumption in using this method is that the component chemical DRC shapes will be the same below the chosen response level [as in DeVito et al. (2000)], attributing the shape differences to high-dose nonlinearities that vary across the components. Differences in DRC shape at high doses are highly plausible due to the potential for saturation of kinetic processes, such as receptor occupancy or metabolic elimination. The choice of the index chemical for application to low response levels, however, should be made carefully because one would probably not want to pick an index chemical with the most extreme shape difference, particularly if only a minor proportion of the other components shared that extreme difference.

In the second approach, the dose-response modeling is based only on data for the lower end of the component DRC, especially important if there is reason to believe that the disagreement across the components is occurring at higher doses, such as by differential metabolism or saturation of receptors. This approach is similar to computing low-response RPFs, except that only the low-dose data are modeled, which could result in a different shape for the DRC than that obtained using the complete data set. This approach was advocated by McGrath et al. (1995), who determined that shape parameter values for several TCDD endpoints computed from data at lower doses differed substantially from those computed from the full DRC. This approach is only possible if there are enough observations in the low-response range for all components to fit a dose-response model. For example, when fitting sigmoid models, there should be enough dose groups so that curvature above and below the inflection point can be adequately described. For the low-response range, where simple non-sigmoidal models should be adequate, there should likewise be enough nonzero doses to allow estimation of curvature (see Table A-1).

4.1.3.2. Methods Based on the Interaction Index Equation

The approaches discussed thus far use the Finney-Bliss definition of dose addition that assumes constant relative potencies, which lead to similarly shaped DRCs and similar models with the consequence that parameters other than dose coefficients are common across the components. The more general definition represented by Berenbaum's equation has no such assumption (Berenbaum 1981). For *n* chemicals, the chemicals are considered dose-additive if the following equation holds for response level *x*:

$$\sum_{i=1}^{n} \frac{d_i}{EDx_i} = 1 \qquad (4\text{-}4)$$

where

d_i = Dose of i^{th} component in the mixture that yields joint response *x*

ED_{xi} = Dose of i^{th} component alone that yields response *x*.

Several approaches have been developed based on the interaction index (Eq 4-4) and have been applied to various combinations of chemicals. Removal of the similar model requirement allows each component to be described by the best model of its data. The most common direct use of the interaction index equation is for evaluating consistency of the mixture data with dose addition, by inserting the mixture component doses in the numerators (where mixture response level = *x*) and the single chemical ED_x doses in the denominators; if the sum = 1 (not statistically different from 1), dose additivity is assumed to hold.

When the goal is to estimate the mixture dose for a specific response level (e.g., the ED_{10} for an untested mixture), the first step is to define "mixture dose." Clearly there can be many different combinations of component doses that produce the same magnitude of response, i.e., the contour lines on the *n*-dimensional response surface for *n* mixture components. For a mixture of known composition, the component fractions are known. To estimate the ED_x for such a mixture along a ray (fixed fractions), Eq 4-4 is first modified by finding the inverse of each component dose-response function and using that inverse in the denominators instead of ED_x, what Howard and Webster (2009) termed Generalized Concentration Addition. Then each component dose in the numerators is represented as the mixture total dose times the component fraction. The equation is then rearranged to solve for the estimated mixture total dose, which by definition must be the mixture dose. The result is the response of *x*, shown in Eq 4-5 as $ED_{x\text{mix}}$. The resulting formula is in the form of the harmonic mean of the component ED_x values. Smyth et al. (1969) is one of the early publications that used dose addition to explore joint toxic action, naming "the harmonic mean" of the component LD_{50} as the way to estimate the mixture LD_{50} and citing Finney (1952) for the harmonic mean formula.

The general harmonic mean formula is:

$$\widehat{EDx}_{mix} = \left(\sum_{i=1}^{n} \frac{\pi_i}{g_i\left(x, \theta_i\right)} \right)^{-1} \tag{4-5}$$

where:

n = Number of components

$\widehat{ED}x_{mix}$ = Predicted mixture dose (sum of component doses) for *x* response

π_i = Dose fraction of i^{th} component (note: doses are not scaled for relative potency)

g_i = Inverse dose-response function[42] for i^{th} component given response level (x) and parameters (θ)

This harmonic mean formula is a simple transformation of the interaction index from a test for additivity into a predictive model for a fixed ratio mixture. The harmonic mean method[43] appears to be a promising approach to the problem of differently shaped DRCs by allowing different component-specific dose-response models. The method has been applied to a variety of in vivo and in vitro systems, either explicitly or implicitly. The European Food Safety Authority

[42]The inverse function gives the dose as a function of the response. E.g., if $y = f(d) = \exp(b*d)$, then $d = g(y) = (1/b)*\ln(y)$. Then $g(y)$ is the inverse of $f(d)$. For dichotomous data, the probability function is solved for the quantile, thus giving the dose associated with a specified probability.

[43]Kortenkamp et al. (2012) show the harmonic mean in their Eq 4, without formally naming it. The method has also been termed the "isobole" method by Bosgra et al. (2009) or, simply (and implicitly), "a definition of dose additivity" [Moser et al. (2005), citing Berenbaum (1985)]. The term "harmonic mean" method is used here to avoid confusion with the actual isobole formula of Loewe and Muischnek (1926).

has adopted this approach as the primary method for assessing dose addition (EFSA 2013; Kortenkamp et al. 2012; U.S. EPA 2016). This method provides a prediction of the total mixture dose at any preselected response level without the need to assess equality of shape, compute relative potencies, or to use the same dose-response function for every component. The harmonic mean formula was developed more explicitly by Svendsgaard and Hertzberg (1994) for threshold estimates, but their derivation is generally applicable to any fixed response level. Altenburger et al. (2000) applied the method to a mixture of 16 phenol derivatives for estimating a full range of ED_x values for x from 1 to 99 (as percent bioluminescence inhibition in marine bacterium *Vibrio fischeri*). Other applications include an integrated addition approach (Qin et al. 2011) and a fuzzy set approach (Mwense et al. 2004). In Appendix A, Section A.3 is a description of the interaction index formula and detailed derivation of the harmonic mean formula.

The harmonic mean formula can be used to estimate the level of response to the mixture for any combination of component doses and is unrestricted regarding the component dose-response functions[44] if those functions can be inverted to generate ED_x values; this property of inversion allows the best fitting dose-response functions to be used for each component. The method can be applied to any response endpoint scenario, dichotomous or continuous. However, when the shapes of the DRCs are judged to be the same, the standard RPF approach is a better option, being more parsimonious and perhaps more representative of the expected response to the mixture, because the harmonic mean method allows the component shapes to be different, which might bias the result. The harmonic mean method can also be used to predict response to the mixture for continuous data, but only if the control values and high-dose maxima (or minima) are the same for all components. In particular, having the response maxima differ across components is a well-known difficulty of risk prediction for combinations of partial agonists (chemicals with differing limits of response) in mixtures (DeVito et al. 2000; Dinse and Umbach 2012; Howard and Webster 2009; Jonker et al. 2005; Lorenzo and Sánchez-Marín 2006; Ritz et al. 2006; Scholze et al. 2014). In such cases, modified approaches can be considered, such as those discussed in Section 4.2.

The harmonic mean method is not always easy to implement. Numerical issues can arise when calculating confidence limits on the ED_x near a response asymptote because the slope of the curve is near zero, so the confidence interval is broad (a range of doses are associated with approximately the same response level). In the low-dose, low-response region, the usual use of approximate standard deviations on ED_x to calculate a confidence interval can produce negative doses for the lower confidence limit. Altenburger et al. (2000) addressed those difficulties by

[44]BMDS dose-response model slope/shape parameters are not restricted to be ≥1.

using the bootstrap resampling technique for estimating confidence intervals around each ED_x (Rosen and Cohen 1995; Scholze et al. 2001).

The harmonic mean method might not apply in some instances where the AOPs are known to be similar. Bosgra et al. (2009) demonstrated that, under some conditions, the harmonic mean method may not be a general solution to the problem of dependence on the choice of the index chemical. They showed that the harmonic mean method failed to predict the response to the mixture for a hypothetical, but plausible, mechanistic model that simulated enzyme inhibition by similarly acting chemicals. The authors introduced complexity into the enzyme metabolic pathway that resulted in nonlinearity between the chemicals after binding to the site of action on the enzyme. The resulting isoboles were curved, rather than straight, as would be predicted by the harmonic mean method. As a result, Bosgra et al. (2009) concluded that a general method for predicting response to the mixture for this scenario (different shape but dose-additive at a specific location on the AOP) has not been established and outlined a practical approach for approximating limits on the response to the mixture. They summarized the issues well, stating in their discussion section:

"In cumulative risk assessment in practice, an approximation of the true cumulative effect[45] may be sufficient. In the case of nonparallel curves, the effect predicted by dose addition depends on the index chemical chosen. Calculated by equivalents of the steeper curved chemical, the predicted effect will be larger than when the less steep curve is used. One should be aware that either prediction will deviate from the true combined effect, which will be somewhere in between these two extremes. Whether this error is something to worry about depends on how far from parallel the curves are and how precise the estimation needs to be. A practical approach could be to calculate the combined effect with both chemicals as index to see how far they are apart." This approach, however, is an index chemical/relative potency model, with all attendant limitations, suggesting caution when used at low response levels.

Even when the inverse functions are well behaved (e.g., one-to-one functions that do not go infinite), they can be subject to similar uncertainty in low-dose extrapolation as the RPF method, such as high uncertainty when the data are sparse or highly scattered at low doses. As stated previously, U.S. EPA has no policy on low-dose risk extrapolation of fitted models for noncancer hazard estimation. Details on implementation of the harmonic mean method and an example are presented in Appendix A.

4.1.3.3. Biologically Based Dose-Response Modeling

Many of the difficulties described above with applying dose addition methods to components with dissimilar DRCs can be avoided or lessened if biologically based

[45]Bosgra et al. (2009) considered chemicals only, not other stressors.

dose-response modeling is employed, a category that includes toxicokinetic and toxicodynamic models. Among the advantages are the ability to quantitatively estimate the error in the additivity formula (whether dose addition or independent action) under different exposure circumstances, such as acute versus chronic exposures, and time-varying versus constant daily exposure levels. Such models are not necessarily easily constructed in a form useful for risk assessment particularly due to the lack of data to properly parameterize. The following key points might be considered when investigating the potential use of these models.

1) Most publications of biologically based models of interactions in joint toxicity have focused on toxicokinetics (Desalegn et al. 2019). Some models are empirical (U.S. EPA 2007b) while others reflect the rates of chemicals entering and leaving specific tissues and organs. These toxicokinetic models have assisted in understanding why the observed joint toxicity deviates from an additivity formula. Examples for binary mixtures include (Simmons 1996): "the role of increased metabolism in non-additive toxicity resulting from temporally separated exposures; the influence of the time interval separating two chemical exposures; and the role of inhibition of metabolism in concurrent exposure to two chemicals."
2) Some toxicodynamic models do exist and their methods should be reviewed because they can give insight into the molecular processes underlying the interactions. For example, El-Masri et al. (1996) described models where both toxicokinetic and toxicodynamic processes are represented for the toxicologic interaction between carbon tetrachloride and Kepone. They only showed graphical depictions, which are often called conceptual models or functional representations (Krishnan et al. 2002); the actual rates (as differential equations) are not presented mathematically, so the quantitative dependence of rates on the applied dose of Kepone is not shown.
3) Most toxicodynamic models are ordinary differential equations that describe rates of change of key constituents over time. Interpreting those models for risk assessment, e.g., so that dose additivity can be evaluated, requires some modification to replace time as a variable by one or more parameters that are constants. Examples based on a fixed time period (e.g., 48 hours for acute exposure) are peak concentration, concentration area under the curve, and average concentration (or other average response measures, such as fraction of dead cells in an organ).
4) In many applications of toxicokinetics and toxicodynamics to dose-response assessment, kinetics and dynamics models are linked but not nested: "TKTD models follow the principle that the processes influencing internal exposure of an organism, summarised under Toxicokinetics (TK), are separated from the processes that lead to damage and effects/mortality, summarised by the term Toxicodynamics (TD)" (EFSA

2018). Based on the evolving state of the science, the view of this strict separation has changed. Toxicokinetic and toxicodynamic events may both be critical to the expression of a health effect.

5) The definitions of "interaction" vary across researchers using these models. For example, some describe competitive inhibition as non-interaction (Bosgra et al. 2009). Others describe it as a common form of interaction (Anand et al. 2005; Cedergreen et al. 2017; Desalegn et al. 2019; El-Masri et al. 1996; Krishnan et al. 2002). In a recently published framework that proposes a taxonomy of interactions from chemical source to exposed population, both dose addition and independent action are included in the general category of interaction (Price and Leonard 2019; Price et al. 2020).

The use of toxicokinetic and toxicodynamic models can provide key details of why joint toxicity might deviate from dose addition or independent action, and how that deviation might depend on different exposure scenarios. For mixture risk assessment, such information is likely to be more accurate and precise than an additivity formula (as long as definitions and assumptions are clearly stated). Instead of the simple distinction between "additivity" and "interaction," these models allow the risk assessor to focus on the predicted mixture toxicity. While promising, more work is needed in applying toxicokinetic and toxicodynamic models to a wide range of mixtures, exposures, and effects before they can become part of the recommended approaches for mixture risk assessment.

4.1.4. Dose Addition for Convergent Pathways

When it has been established that the initial steps (e.g., MIEs; early KEs) in an MOA leading to a specific common health outcome are independent for mixture components, the use of response addition may not apply. Specifically, if the initially independent pathways merge at a common KE or process before eliciting the adverse effect, dose addition may be appropriate (see Figure 3-4 for a graphical depiction). This concept has been previously illustrated in the literature in the context of receptor theory (Leff 1987; Mackay 1981; Scaramellini et al. 1997) and further developed by Jonker et al. (2005), but only in the context of drug interactions, rather than conditions allowing for application of dose-additive methods. An example of this scenario is the action of endocrine disruptors on androgenic pathways leading to male reproductive effects (e.g., NRC 2008). It is possible that endocrine disruptors that initiate their actions at different receptor-dependent or independent MIEs exhibit their joint toxic action on male reproductive effects by ultimately impinging on a common critical event or process such as decreased androgen-dependent signaling at/in a given target tissue (e.g. Kortenkamp and Koch 2020). In

such cases, the joint action appears to be consistent with dose addition rather than response addition. This is because the common KE for androgen-dependent disruption of reproductive development is reduced signaling in respondent tissues, although by different mechanisms (e.g., some disruptors inhibit steroid biosynthesis while others may inhibit androgen receptor-ligand binding). Regardless of the specific pathway(s), the reduced testosterone and/or androgen receptor-dependent signaling then leads to a variety of male reproductive effects in rats. In this scenario, dose additivity does not occur at the initial chemical/tissue interaction level (the MIE) but rather at a downstream pathway level (e.g., androgen-dependent signaling events). This scenario can also be envisioned for other endocrine-driven endpoints like thyroid toxicity or hormonally driven female reproductive effects. There may be some constraints on the system, such as similar dose-response shapes or proportional intermediate processes, but these have not yet been established. Scaramellini et al. (1997) found very narrow conditions under which pure dose additivity applies for a two-receptor, one-transducer (common process) mathematical model simulation applicable to the convergent pathway scenario, but at this point it is unclear whether and how far their results can be generalized.

4.2. ISSUES IN USING DOSE-ADDITION METHODS WITH CONTINUOUS OUTCOMES

Although much of Section 4.1 applies to both dichotomous and continuous endpoints, additional factors need to be considered for continuous response data. The two critical issues specific to continuous measures, noted in the introduction to this section, are differences in control values and in maximal effect ("partial agonism"), both of which are discussed below. Dose-response modeling of continuous endpoints for risk assessment is made more difficult than for dichotomous outcomes because there is no natural probability scale with which to characterize risk. If the mixture components do not have the same response limits (control mean and high-dose plateau[46]), dose-additive modeling becomes more complicated. Even when dose-response shapes are the same, use of nominal RPFs could result in inaccurate risk predictions (Chen et al. 2003; Dinse and Umbach 2011; Jonker et al. 2005). Dinse and Umbach (2011) stressed that one must first determine whether the differences are due to the chemical itself ("intrinsic;" e.g., maximal chemical effect) or to factors independent of chemical exposure ("extrinsic;" e.g., control values). Intrinsic differences must be included explicitly in the dose-addition model, while extrinsic differences potentially can be ignored (if minor) or factored out by subtraction or scaling/normalization.

[46]Largest or smallest response, depending on the direction of the change in response with increasing dose.

Control values are obviously extrinsic, and conceptually both the mean and variance are the same for the same endpoint in the same animal model.[47] Control values, however, can vary, even under identical bioassay protocols. For example, control means for the 14-week liver levels of ethoxyresorufin-O-deethylase (EROD) activity in the NTP bioassays for DLCs varied fourfold, while the standard errors varied twofold; the 31-week lung EROD activity control means and standard errors both varied about 2.6-fold (NTP 2006a; 2006b; 2006c; 2006d). Other continuous endpoint control values were significantly different as well. If the control means or variances for the different components of the mixture are not the same, mixture risk prediction based on the endpoint absolute values then depend on the control mean and the variance of the selected index chemical (Chen et al. 2003). A difference in control variances will result in a mixture risk prediction that depends on the choice of the index chemical when the response measures are scaled to the control mean (Chen et al. 2003). When confronted by significantly different control values, the assessor can determine the most representative value on a case-by-case basis. If there is no identifiable extrinsic factor affecting one or more of the control animals (e.g., infection or illness), normalizing the measured response values to the control group is a common practice. Normalizing the responses to control means, either by subtraction or by scaling (Moser et al. 2005), eliminates the dependence on the control mean, but does not resolve the issue of different control variances. Depending on the magnitude of the differences, the representative value for the variance could be the mean, median, geometric mean, or simply a "best" value; no practical guidance on the choice of approach can currently be given, but the assessor might consider the spread of the values and the relative quality of the studies.

Differences in maximal chemical effect (high-dose extrema [either maxima/minima or "asymptotes"]) could be intrinsic (a toxicological property of the chemicals once inside the body) or extrinsic and are more difficult to assess than are control differences. Extrinsic factors could be a result of random error or systematic differences among assays, in which case they need to be addressed by adjustments to the response data. For example, if there is a shift in the control values similar to the difference in the high-dose maxima, an extrinsic source could be considered, and the response data adjusted to match the index chemical range. However, lacking good evidence that extrinsic factors are involved, the differences logically would be considered intrinsic. The phenomenon of partial agonism (differences in response asymptotes at high dose) is commonly encountered in studies in which toxicity is predominately driven by receptor-dependent pathways (Hestermann et al. 2000; Peters et al. 2006); some agonists in a mixture may not elicit 100% receptor activity at saturation levels. Partial agonism has also been reported for phytotoxicity (Belz et al. 2008; Ritz et al. 2006), in vitro hormonal activity

[47]The same measured endpoint in the same species, strain, and sex. However, the concept can be generalized to any specific test system, in vivo or in vitro.

(Rajapakse et al. 2004; Silva et al. 2007), and animal bioassays (Crofton et al. 2005; Moser et al. 2005; NTP 2006a; NTP 2006b; NTP 2006c; Rider et al. 2008). The phenomenon has commonly been described under experimental exposure conditions where high(-er) concentrations or doses of chemical(s) are employed to force receptor occupancy to saturation. The applicability of receptor saturation in environmental exposure scenarios, however, is not anticipated, as the influence of partial agonism in human health systems is unclear. Further, as a practical matter for dose addition, predicting response to the mixture above the lowest partial agonist maximum response is problematic, because there is no dose for the partial agonist corresponding to more extreme responses. That is, no matter how high the partial agonist dose becomes, the response will never get larger.

A few published approaches can be tried as a solution to the partial agonism issue (Dinse and Umbach 2011; 2012; Gennings et al. 2004; Howard and Webster 2009; Scholze et al. 2014). Gennings et al. (2004) described a model for use with partial agonists[48] that is a form of the harmonic mean approach that also allows for component-specific toxicity threshold doses. This model was used subsequently in several papers reporting departures from additivity where the responses were scaled to the response range for each component (0–1 transformation) (Crofton et al. 2005; Moser et al. 2005). However, Ritz et al. (2006) cautioned that the transformed data are not directly comparable to the original measures at all response levels. That is, normalizing the continuous response measures to their respective ranges results in a dependence on the choice of a reference range (i.e., reference chemical) if transformation back to the original units is desired; under a 0–1 transformation, a percentage change for a partial agonist does not return the same value of the original measure as for a full agonist.

Howard and Webster (2009) presented an approach for modeling partial agonists, the generalized concentration addition (GCA) model mentioned earlier, which was used by Howard et al. (2010) for modeling the joint effects of aryl hydrocarbon receptor agonists with partial agonists and competitive antagonists. In the GCA model, the response data are scaled to the overall maximum response (of the full agonist) representing partial agonist maxima as a fraction of the overall maximum. Importantly, in the GCA application, the inverse functions are not restricted, thus allowing a "virtual ED_x" of the partial agonist that is negative. Their GCA example was for ligand binding using a relatively simple dose-response function (i.e., a Hill function with exponent = 1) but the general approach allows more complex models. Hadrup et al. (2013) applied the GCA to mixtures of estrogenic chemicals in an in vitro assay and were able to generate a reasonable full DRC for two different chemical mixtures (including responses above

[48]Equation 6 in the appendix of their paper.

the lowest partial agonist maximum), but they reported that a single chemical tended to dominate in both mixtures.

Scholze et al. (2014) described a model for the high response region of the DRC that includes a partial agonist maximum "toxic unit" contribution to the mixture risk prediction above the partial maximum response. That is, an assumption was made that at doses above saturation for the partial agonist, that chemical's contribution to the response to the mixture will not go to zero but will remain at some relatively constant level, with extremes from zero to some subjectively determined higher level. The toxic unit is defined as the chemical concentration (dose) divided by the ED_{50}, which makes this an implicit RPF model,[49] but without an explicit index chemical. The maximum toxic unit contribution of partial agonists to the response to the mixture in the Scholze et al. (2014) formulation was computed at the ED_{70}. The ED_{70} was not a strictly mathematically rigorous cutpoint but required judgment on the balance of over- or under-representation of the chemical contribution at doses above its partial maximum. In this model, as in the GCA, the response data are scaled to the overall maximum response of the full agonist, rather than including in the model a parameter for the range of responses, as in the Gennings et al. (2004) model. The model was tested with a set of estrogenic compounds in an in vitro assay and performed reasonably well above the lower partial maxima.

Another approach, developed for single-chemical dose-response assessment, but not yet applied for mixtures prediction, is dichotomization of the continuous measures for analysis with probabilistic methods. This approach estimates the incidence of individuals falling above or below a response level considered to be adverse. Thus, it offers a solution to the partial agonist problem by converting the continuous data to response probabilities, such that the methods described for dichotomous data in Sections 4.1 and 4.2 can be applied. Implementing the approach requires setting a specific response level as an adversity threshold, which can require substantial scientific judgment, or setting standardized adversity levels (e.g., 95th percentile of the response range). In addition, information is lost in the conversion process (West and Kodell 1999).

U.S. EPA has not yet rigorously evaluated these approaches for chemical mixtures risk assessment. It should be noted that biological pathways leading to human health outcomes may be diverse but also may appear to be coordinated (i.e., connected) at the level of the outcome, with complexities beyond the concerns about partial agonists. One caution mentioned previously for 0–1 scaling (see Section 2.1.5) is that if lower or upper limits differ across components, normalizing responses to those limits can result in inaccurate or biased mixture risk predictions (Dinse and Umbach 2012; Ritz et al. 2006). The degree of potential bias for varying magnitudes

[49]RPFs are often calculated as the ratio of ED_{50}s.

of difference in the limits has not yet been investigated. In these cases, the assessor may want to limit prediction of response to the mixture to lower dose ranges, in which individual chemical doses are well below their respective high-dose-response limits.

4.3. UNCERTAINTY ANALYSIS OF RELATIVE POTENCY FACTOR APPLICATIONS

Application of the RPF approach is straightforward when there are official sets of RPFs available, such as the TEF values for the dioxins. Recent advances in RPF concepts and calculation are more complicated and reflect many previously unstated uncertainties (see discussion in Section 1). The U.S. EPA Supplementary Guidance on mixture risk (U.S. EPA 2000c) recommended the characterization of uncertainties in any mixture risk assessment. The findings of this White Paper are consistent with those recommendations, as well as several considerations with probabilistic methods described in U.S. EPA (2014c). These considerations may be particularly relevant when developing a quantitative uncertainty analysis for RPFs or when applying RPFs in a risk assessment. The U.S. EPA guidance stated that uncertainty analysis "is crucial to proper interpretation of the RPF approach and the resulting mixture risk assessment." The areas of qualitative and quantitative uncertainty pertaining to RPFs and their application could include, but are not necessarily limited to, the following:

- Evaluating the uncertainty and variability of the index chemical data (e.g., for hazard identification and dose-response) and the data supporting RPFs for the other chemicals. That evaluation could include the confidence in the qualitative and quantitative data used in the calculation of component RPFs and the relevance of those data to the assessment purpose, with any differences noted of the RPF quality across the chemicals (see Section 2, specifically Section 2.2.5).
- Defining the limits on the scope of application of the RPFs (e.g., the health endpoints, exposure routes, exposure duration, and dose range covered) (see Section 2.2.5).
- Examining the numerical consistency of the RPF across the compounds considered and across different scenarios (e.g., whether RPFs are considered applicable to multiple health endpoints, exposure durations, dose range, or multiple exposure routes). This examination also could include consistency of RPFs when based on different points on the DRC (e.g., RPF estimates based on ED20s could differ from RPF values based on ED_{10}s, suggesting the DRCs of the chemicals are not similar). Whenever alternative approaches described in Section 4.1.3 are to be considered, such consistency evaluations could provide the empirical rationale or at least the uncertainty characterization of the chosen approach.

- Evaluating the quality of evidence supporting toxicological similarity (see Sections 3 and 5).
- Evaluating the extent of support for dose additivity based on inferences from previous toxicology tests of reasonably similar mixtures.

Several structured approaches have been published for both qualitative and quantitative uncertainty analysis that could be useful for RPF applications. Some are general U.S. EPA guidance for probabilistic methods for risk assessment (U.S. EPA 1997; 2001c; 2014b; 2014c). Some have been developed and evaluated specifically for epidemiological data (Czarnota et al. 2015; Keil et al. 2020). Others have been developed or demonstrated for new approach methodologies (NAMs) assays (discussed in Section 5). Because RPFs involve a ratio of two estimated quantities, each of which is derived from a dose-response analysis, the quantitative uncertainty characterization for RPFs can require advanced methods and resources. The U.S. EPA report of Frequently Asked Questions for probabilistic risk assessment has several ideas on how to decide if the probabilistic risk assessment effort justifies the required resources (U.S. EPA 2014b).

5. FUTURE ROLE OF 21ST CENTURY TOXICITY TESTING AND NEW APPROACH METHODOLOGIES IN CHEMICAL MIXTURES RISK ASSESSMENT

5.1. CHALLENGES FOR 21ST CENTURY MIXTURE ASSESSMENT

Conventional mixture assessment of environmental chemicals has typically relied on the availability of apical effect information obtained from traditional animal studies (e.g., 90-day or 2-year bioassays) and/or human epidemiological exposure-response data. However, a pervasive challenge in conducting mixture assessment is the lack of useful hazard and dose-response information for the relevant environmental mixture of interest (e.g., parent chemicals and/or degradation products in the exposure media at environmentally relevant mixture proportions) and/or the individual chemicals comprising the mixture. Because toxicity information is generally lacking for the thousands of chemicals found in environmental media across the globe, a revolution in toxicity testing began in the early 2000s. For example, in response to a request by the U.S. EPA, the National Research Council (NRC) reviewed a broad landscape of toxicity testing methods and approaches, and developed the seminal publication *Toxicity Testing in the 21st Century: A vision and a strategy* (NRC 2007). The NRC committee recognized at that time "The current approach to toxicity testing relies primarily on a complex array of studies that evaluate observable outcomes in whole animals, such as clinical signs or pathologic changes that are indicative of a disease state. Partly because that testing strategy is so time consuming and resource-intensive, it has proved difficult in meeting many challenges and needs encountered in human health risk assessment, such as evaluating various life stages, numerous health outcomes, and large numbers of untested chemicals" (NRC 2007). While these traditional testing approaches have required significant time and resources, they have served well the practice of regulatory toxicology; indeed, test animal data are the basis of many U.S. EPA human health toxicity values. Since the inception of the NRC strategy, toxicity testing has been shifting increasingly from resource-intensive (e.g., large numbers of test animals; multiyear study designs and assays, and often complex data acquisition and interpretation/evaluation) to cost-saving, animal-sparing, and shorter duration assays that inform the biological activity of environmental chemicals (Krewski et al. 2020; Thomas et al. 2019; U.S. EPA 2020).

High-throughput platforms such as -omics (e.g., transcriptomics, proteomics, metabonomics), chemo- and bioinformatics (e.g., structure-activity relationships, read-across), systematic survey and review of extant toxicity information (i.e., study/data mining techniques), cell-based bioactivity assays (e.g., receptor-ligand binding/activity, enzyme activities), reverse toxicokinetics (e.g., in vitro to in vivo extrapolation [IVIVE]), and nonmammalian alternative whole organism testing models (e.g., *Drosophila, Caenorhabditis elegans,* zebrafish) have all been proposed for expediting human health risk assessment (Collins et al. 2008; Cote et al. 2016;

Huang 2016; Kavlock and Dix 2010; Perkins et al. 2013; Richard et al. 2016; Thomas et al. 2019; U.S. EPA 2020). For brevity, these various high-throughput/high-content approaches will be referred to collectively as new approach methodologies (NAMs). In a risk assessment context, NAMs are defined as any technology, methodology, approach, or combination that can provide information useful for risk assessment (including hazard assessment, dose-response assessment, and exposure assessment) without the use of traditional test animals (e.g., rats, mice), including in silico, in chemico, in vitro, and ex vivo approaches (ECHA 2016; U.S. EPA 2018b). Each type of NAM comes with advantages and challenges for application to hazard identification and dose-response assessment for chemicals; however, the complementarity of kinetic and/or dynamic information derived from NAMs could be transformative once implemented. Specifically, not all risk assessment problem formulations or foci necessitate the same type, level, or rigor of toxicity data to support some action or conclusion. For example, the body of evidence needed for preliminary screening of potential hazard of chemicals may differ significantly from that needed for identification of quantitative points of departure (PODs) for derivation of human health toxicity values in a formal risk assessment. This is not necessarily unique to the use of NAM data, as opposed to traditional human epidemiological or experimental animal assay data; however, at this time Agency-wide guidance has not been finalized and published that codifies or frames application of NAMs in various risk assessment contexts. Although not guidance per se, a European initiative, referred to as "EuroMix", has framed a gradation of mixture application domains for NAMs including: (1) Determine a refined grouping strategy for assessment purposes; (2) Identify criteria for prioritization of chemicals for mixture testing; (3) Evaluate qualitative and quantitative concordance between results from in silico methods and in vitro bioassays against in vivo animal testing data; (4) Establish best practices for extrapolation of in vitro bioassay and in silico model results to humans; and (5) Develop harmonized tools and models for assessment of chemical mixtures (Di Renzo et al. 2019; Heusinkveld et al. 2020; Lichtenstein et al. 2020; Rotter et al. 2018). These objectives comport with targeted NAM-based testing and assessment foci in U.S. EPA.

While the use of data from NAM studies has not routinely been considered in regulatory risk assessment decision contexts, and chemical mixtures are not currently a key focus area in 21st century toxicity testing, the potential for individual chemical data from NAM platforms to inform mixtures risk assessment is high. In particular, the use of NAM data for single chemical evaluation(s) in U.S. EPA demonstrates application domains that also will be applicable for mixtures risk assessment. For example, for the evaluation of data-poor chemicals (for an example, see Appendix A of the U.S. EPA Provisional Peer-Reviewed Toxicity Value assessment for p,p'-DDD), structure-activity/read-across has been applied for over 30 human health assessments to infer or interpolate hazard and dose-response information from more

data-rich chemicals. Another recent example is the development of an approach that leverages pathway-based transcriptomic data in the derivation of human health toxicity values for data-poor chemicals. The EPA Transcriptomic Assessment Product methodology has undergone peer review by a Board of Scientific Councilors, external to U.S. EPA, and could become part of a portfolio of human health assessment products. Additional NAM application efforts are in an exploratory phase of development but are intended for application to diverse decision contexts for single chemical evaluations. Single chemical NAM applications in U.S. EPA, to date, inherently demonstrate potential for mixture assessment application. However, until more effort, resources, and focus are placed on defined mixtures studies, formal mixture assessment will likely continue to be reliant primarily on single chemical hazard and dose-response data, using component-based mixtures approaches (U.S. EPA 2000c). Further, as with traditional in vivo assay-based toxicity data, there are qualitative and quantitative uncertainties associated with NAM data. There are several complexities associated with NAMs, such as in vitro cell-based assays, that are unique compared to traditional experimental animal bioassays; these include (but are not limited to) stability/volatility of chemical in in vitro systems, metabolic competence of cells in culture, data acquisition conditions (e.g., signal to noise ratio across levels of biological organization), and qualitative and quantitative relationship of results to apical effects/health outcomes. There are additional considerations associated with in silico NAMs (e.g., predictive toxicity; read-across) such as chemical diversity (or lack thereof) in the training set on which a given model was trained, criteria for determination of similarity (e.g., structural, physicochemical, kinetic) between two or more chemicals/analogues, and quantitative precision or concordance of NAM-based data to apical effect-based data. These considerations are not isolated to mixture risk assessment but rather are inherent areas of uncertainty associated with NAM-based chemical evaluation in general.

A comprehensive treatise on NAMs and the various chemical evaluation application domains are not the objective of this document; however, the reader should be aware of the immense potential for more rapid data generation that could inform chemical mixtures risk assessment. Sections 2–4 of this document have already discussed the methods and introduced important details, considerations, and nuances associated with qualitative and quantitative evaluation of toxicity data for informing mixtures approaches based on dose addition, so will not be repeated here. Considering the paucity of traditional toxicity data for the vast majority of environmental chemicals, the integration of NAMs to inform decisions regarding health outcome weight of evidence (WOE), mode of action (MOA)/adverse outcome pathway (AOP) membership, and dose-response characteristics (e.g., dose additivity, response additivity, or deviations from additivity) will be paramount in advancing mixture risk assessment. Almost every NAM approach or platform proposed to date may provide evidence for qualitative

biological membership (i.e., operative in a given health effect domain and/or MOA/AOP); many NAMs also can provide empirical quantitative dose-response data that might inform identification of PODs (e.g., cell-based bioactivity; short-term in vivo transcriptomics), or at least help contextualize such data for risk assessment purposes (e.g., high-throughput toxicokinetics; IVIVE). While this document is not intended to provide an overview of NAM platforms, in general, it is critical to note that reverse dosimetry approaches for converting in vitro concentrations to an approximated equivalent human exposure dose are paramount for use of data derived from toxicogenomic study designs (e.g., cell-based bioactivity assays) in risk assessment (Bell et al. 2018; Chang et al. 2022; Wambaugh et al. 2018; Wambaugh et al. 2015). In the most data-poor scenarios, some NAMs, such as (quantitative) structure-activity relationships/read-across, can provide a means to leverage hazard and dose-response data from more data replete chemicals as surrogate for data-poor (similar) chemicals. As can be imagined, dependent on the mixture, there may be a diverse landscape of component chemicals with varying data needs to inform proper component-based mixture assessment.

One of the initial steps in any component-based mixture assessment is grouping individual chemicals based on their toxicological similarity, one of the two grouping characteristics articulated in Section 3. Groups exhibiting a toxicological or biological commonality have been described previously as sharing a common adverse outcome (AO) (NRC 2008), a common MOA (U.S. EPA 2000c; 2005a), and/or a common mechanism group (U.S. EPA 2002a). Individual chemical pathway perturbation and/or molecular and cellular "outcome" data from NAM platforms may facilitate predictions of toxicity anticipated from a mixture exposure in the virtual absence of traditional apical toxicity data. Some key component hazard-identification and dose-response assessment questions for mixtures that data from NAM may inform include the following:

- Do chemicals "look similar" to one another (e.g., structurally and/or physicochemically);
- Do they share the same or similar profile of absorption, distribution, metabolism, and/or elimination (i.e., toxicokinetics);
- Do they share or induce the same or similar bioactivities; and ultimately,
- Do they result in (if even just a prediction) a similar adverse health outcome?

Virtually every NAM platform currently available can inform one or more of these basic queries about grouping membership and component-based mixture assessment.

As described in Sections 2–4, a primary assumption underlying dose addition is that the responses to chemicals share the entirety of a MOA/AOP, or minimally, share one common key event (KE; could be toxicodynamic and/or toxicokinetic) in a response pathway that follows

from the molecular initiating event (MIE) to the apical health outcome. For some health endpoints, for chemical classes such as dioxin-like compounds that operate through highly defined aryl hydrocarbon receptor (AhR)-mediated pathways, a common MOA is strongly supported. In contrast, for the diverse universe of mixture chemicals operating though complex networks of biological activity (i.e., multiple different MIEs and/or receptor-independent perturbations), there may be a default assumption that such chemicals would not be dose-additive. This is particularly difficult in a NAM data context due to the biological granularity at which hazard and dose-response are described/characterized. Data may be available to evaluate whether the chemicals share the entire toxicodynamic pathway, from MIE to the expression of the apical health outcome, or only one or a few KEs are shared by different but merging pathways (see Figure 5-1). As noted in Section 3, when examining similarity among mixture components, the evidence can vary widely along a biological continuum; this chapter articulates an approach that may facilitate inclusion of individual chemical evidence derived from NAMs (see Section 5.2). In cases where dose additivity is shown not to hold (e.g., two chemicals cause the same apical outcome through independent response pathways), other mixtures methods, such as response addition or specific interaction models, could be considered along with, or instead of, dose addition.

Once chemicals are qualitatively grouped together, the greater difficulty is determining whether and how the NAM-based concentration-response data can be used to inform the hazard characterization or mixtures dose-response assessment steps. Interpreting how a given level of perturbation in a nonapical effect (e.g., an in vitro bioactivity concentration that is 50% of maximum response, referred to as an AC_{50}) translates to incidence and/or magnitude of adverse phenotypic health outcome(s) in a living organism remains a challenge and a source of uncertainty when using such data in risk assessment (Paul Friedman et al. 2020). Further, trying to make sense of atypical concentration-response characteristics is currently both challenging and uncertain. For example, some mixture chemicals may have NAM data with bimodal or other forms of nonmonotonic/irregular concentration-response curves or, sometimes, have no useful concentration-response information at all.

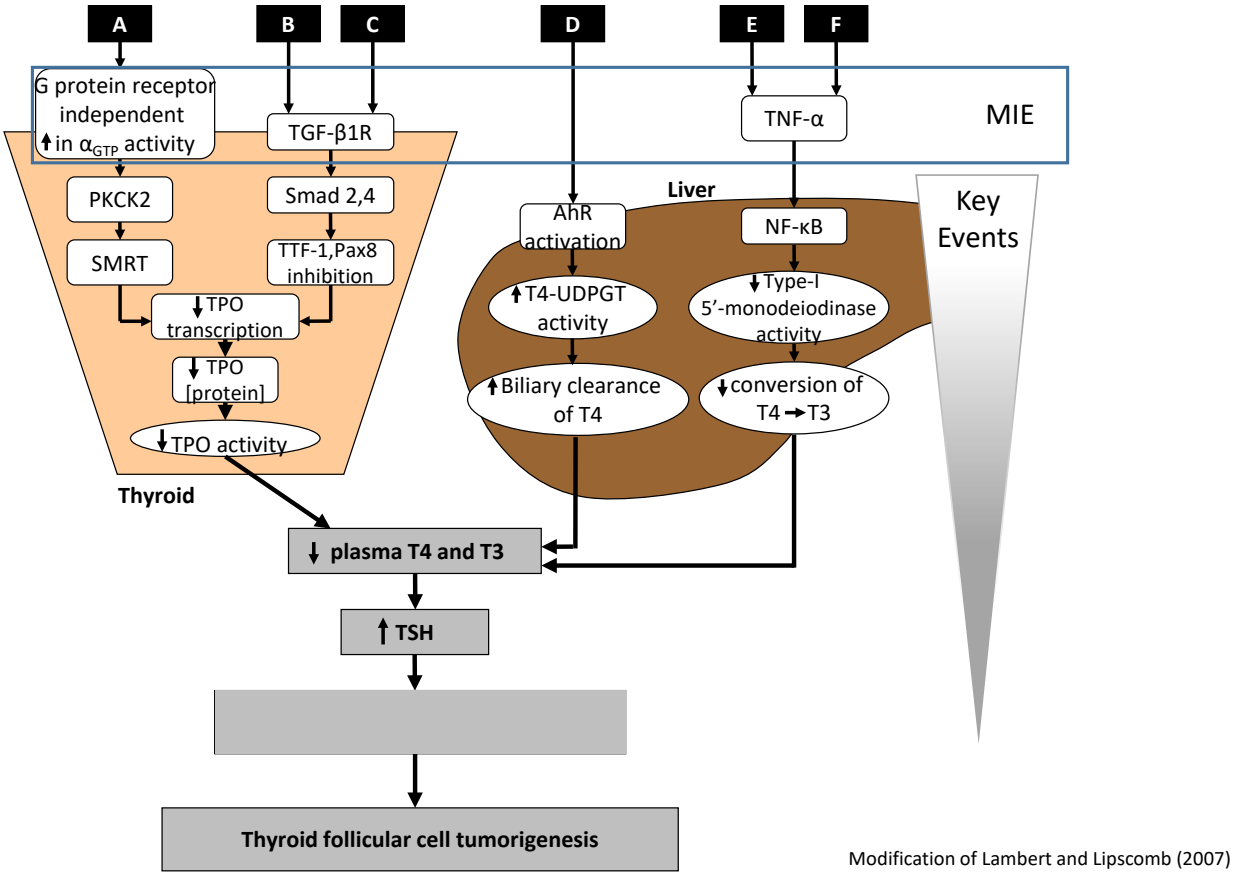


Figure 5-1. A hypothetical mixture of six chemicals induces thyroid follicular cell tumorigenesis via several related adverse outcome pathways.

While there is no apparent similarity across all chemicals at the MIE level, evaluation of potential KEs along AOPs reveals a convergence point/KE (e.g., decreased plasma levels of thyroid hormones T4 and T3) where all causal AOPs affect the same apical outcome through the same downstream KE.
AhR = aryl hydrocarbon receptor; AOP = adverse outcome pathway; GTP = guanosine-5′-triphosphate; KE = key event; MIE = molecular initiating event; NF-κB = nuclear factor kappa B; Pax8 = paired box 8; PKCK2 = protein kinase casein kinase 2; Smad 2,4 = mothers against decapentaplegic homolog 2 and 4; SMRT = silencing mediator of retinoic acid and thyroid hormone receptor; T3 = triiodothyronine; T4 = thyroxine; TGF-β1R = transforming growth factor beta-1 receptor; TNF-α = tumor necrosis factor alpha; TPO = thyroid peroxidase; TSH = thyroid stimulating hormone; TTF-1 = thyroid transcription factor 1; UDPGT = uridine 5′-diphospho-glucuronosyltransferase.

In applying NAM information to mixture assessment, analysts should recognize that environmental chemicals were/are not typically designed or intended to specifically interact with biological targets in humans. Exposure to an environmental chemical may result in activation and/or inhibition of a diverse array of receptor-dependent and receptor-independent pathways that lead to complex bioactivities and ultimately to a host of phenotypic adverse health outcomes. The myriad kinetic and dynamic pathway perturbations that orchestrate an adverse response have been evaluated for human health assessment purposes under several related constructs, such as mode or mechanism of action (see Section 2.1.1). However, the toxic

mode/mechanism of action concept has subsequently been integrated and expanded under the AOP paradigm (OECD 2013). AOPs were first developed to characterize toxicity pathways (TPs) in ecological species (Ankley et al. 2010) but have now been posited as the modality for structured qualitative, and potentially quantitative, descriptions of causal events spanning the entire exposure-to-outcome continuum, from MIE up to population-level dynamics for humans and ecological species (Edwards et al. 2016; Villeneuve et al. 2014a). While many environmental chemicals induce complex and seemingly chaotic biological perturbations at a mechanistically granular level of organization, AOPs are represented commonly as unbranched (i.e., linear) "if this, then that" flow of causal nodes or KEs (Villeneuve et al. 2014a). A more recent conceptualization of AOP networks takes into account the complexity and inter-relatedness of AOPs that affect phenotypic expression of a given health outcome (Knapen et al. 2018; Villeneuve et al. 2018). A key caution is that AOPs inherently illustrate toxicodynamics but not toxicokinetics. Thus, an MIE is identified typically as the earliest toxicodynamic event in an AOP (e.g., interaction of a chemical with a receptor). However, mixture chemicals could impact or influence one another through kinetic processes. For example, one chemical might upregulate the expression of enzymes responsible for Phase I (i.e., CYP450s) and/or Phase II (e.g., conjugation reactions such as glucuronidation, acetylation, and sulfation) metabolism of another mixture component chemical. Importantly, these kinetic interactions may occur anywhere along the source to health outcome continuum in a given AOP. The importance of kinetic considerations within the context of an AOP/AOP network is that some mixture components may materially alter the qualitative dynamics and/or dose-response of another component (or potentially itself) in a given target tissue (see Figure 3-4). If a toxicokinetic activity, process, or event is deemed "key" for an AOP, then it should be explicitly identified as such.

The placement of mixture chemicals into same/similar or dissimilar groups has been evaluated historically at the apical end of an outcome pathway (i.e., organ/tissue level effect). The advantage of this approach is that it is relatively straightforward to determine from empirical dose-response studies. Challenges in the approach include the lack of existent apical effect information for many chemicals found in mixtures of interest, increased focus on reducing animal use in toxicity testing, and the absence of an accepted framework or approach for establishing "adversity" of precursor or upstream effects in a given AOP. With an increasing focus on evaluating hazard and dose-response further upstream from the apical outcome, particularly for chemicals for which data are limited, integrating individual component-based information from NAMs in a mixtures context could potentially complicate hazard interpretations. The analyst could find data on a diversity of cellular/molecular signaling pathways for each chemical, and thus might interpret the data as indicating deviation from dose

additivity (i.e., chemicals may not look similar simply because of the inherent diversity of bioactivity at a mechanistic level of "hazard" understanding) (Lutz et al. 2002). Thus, the key to "hazard" grouping of chemicals is the dose and AOP process at which decisions regarding commonality are made. While toxicodynamic (and/or kinetic) pathways or networks are typically diverse and complex downstream of an MIE, it has been observed empirically that there are critical molecular/cellular junctions that serve as a convergence point for signaling and that these junctions are often a KE proximally located to an AO. A prime example was illustrated in the National Academy of Sciences NRC report in 2008 titled *Phthalates and Cumulative Risk Assessment: The Tasks Ahead* (NRC 2008), in which several potential adverse reproductive/developmental health outcomes in males (primarily experimental rats) were proposed to occur via a number of different pathways (see Figure 3-4 of the 2008 NRC report). While the mechanistic details proceeding from the various MIEs (e.g., androgen receptor blockade or decreased CYP11A1/CYP17A1 levels or activity) entail unique bioactivities (Rider et al. 2010), the pathways ultimately converge at the same downstream junction or KE, for example, decreased androgen receptor activation at the target tissue. Thus, in the absence of apical-effect-level information, leveraging other biological information could inform selection of critical intermediate KEs for health outcomes associated with mixture exposure(s), which may contribute to evaluations of dose additivity (Conley et al. 2016; Watt et al. 2016; Wegner et al. 2020).

A major challenge within the risk assessment community is the lack of a common lexicon for describing the pathway(s) by which chemicals induce toxic effects. For example, many assessment practitioners adhere to the concept of MOA, which is defined as a sequence of KEs and processes, starting with the interaction of an agent with a cell, proceeding through operational and anatomical changes, and resulting in a toxic effect [e.g., cancer formation; U.S. EPA (2005a)]. MOA is contrasted with *mechanism of action*, which to some implies a more detailed understanding and description of events, often at the molecular level, than is meant by MOA. More recent efforts in the AOP project have expanded TP annotation from an MIE to AOs at the exposed population level (Ankley et al. 2010; Edwards et al. 2016; Villeneuve et al. 2014a). Although there are some nuances to AOP, MOA, and mechanism of action, the shared vision is description of the toxicodynamic and kinetic events linking exposure to a target site and the resultant health outcome(s). An AOP could be viewed as the umbrella construct under which the mode and mechanism of action are a subset of pathway descriptors. Recall that MOA and mechanism of action are often associated with specific chemicals or classes of chemicals, whereas AOPs are supposed to be chemical agnostic. Causal nodes or events in an AOP commonly rely on multiple lines of evidence across assay types, experimental species, sexes, and exposure durations irrespective of the chemical(s) that underpin such bioactivity information.

Regardless of the level of biological organization at which a given TP is described, a critical unifying factor of AOP, MOA, and mechanism of action is the KE. A KE is an empirically observable precursor step that is itself a necessary element of the MOA or is a biological marker of such an element (U.S. EPA 2005a). As such, while the level of biological detail used to define causal nodes/events in a TP may differ across chemicals and assays, the concept of a "key event" is conserved; even the most detailed mechanistic step could be considered a KE if it is a necessary step in the pathway to an adverse health outcome. The critical importance of KE information in the context of NAM data application to the evaluation of dose additivity for mixture assessment will be discussed in Section 5.2. For brevity, further reference to AOP is meant to also encompass both MOA and mechanism of action.

The issues and considerations highlighted above are just a small sample of the many complexities associated with integration of NAM data into mixture assessment. Section 5.2 introduces opportunities for NAM data in informing qualitative hazard and dose-response assessment for chemical mixtures using a structured but flexible approach.

5.2. OPPORTUNITIES FOR NEW APPROACH METHODOLOGIES AND DATA IN MIXTURE ASSESSMENT

NAM platforms offer the prospect of generating a tremendous "landscape" of structural, physicochemical, and molecular and cellular bioactivity information, particularly for chemicals with little or no traditional hazard data available. However, as with apical outcomes, the limited availability of mechanistic or pathway-based mixtures studies hampers evaluating the additivity of chemicals. The current reality is that NAM data will be generated primarily for individual chemicals, with component-based mixtures methods key for predicting mixture toxicity. However, as discussed in Section 5.1 above, application of nonapical NAM data in mixtures dose-response assessment may be challenging without flexibility in interpreting what constitutes a hazard. For example, the grouping of "common" chemicals may differ as a function of mechanistic granularity. It is one thing to say that two or more chemicals belong to a common grouping based on evidence of reduced thyroid hormone (e.g., T3 and T4) levels, but interpretations may be entirely different if more upstream AOP data identified that one chemical causes a reduction in thyroid peroxidase activity and the other chemical induced tumor necrosis factor-mediated thymocyte cell death (see Figure 5-1). In the former, a qualitative interpretation of "common" is likely; in the latter, an AOP/MOA-based determination of dissimilarity may be more likely, although both upstream KEs likely contribute to a decrease in thyroid hormone levels. Further, concentration-response characteristics among "common" chemicals in vitro may be diverse (e.g., different slopes of concentration-responses) suggesting deviation from dose additivity when indeed they should be dose-additive based on common KE(s). In addition,

assessing the developmental toxicity of chemical mixtures using NAM data may not reflect the role of lifestage considerations such as critical time windows of exposure. Integrated NAM approaches (OECD 2013; Patlewicz et al. 2014; Tollefsen et al. 2014) may help facilitate incorporation of diverse hazard and concentration-response information to better inform qualitative chemical grouping as well as to select the most appropriate additivity approach.

While NAM data will significantly augment our understanding of qualitative hazard, such as mixture component similarity groupings, the utility of concentration-response information from these assays in a human health mixture assessment application is unclear. For example, a tremendous challenge for NAM data to date has been causal linkage of nonapical perturbations to apical outcomes. That is, associating the magnitude of change at a molecular or cellular level to some phenotypic effect comes with a diverse array of complex considerations that has significantly limited translation or application of NAM data to dose-response assessment. Traditional toxicity studies already have many variables to consider in dose-response interpretation such as species, strain, sex, life stage, duration of exposure, route of exposure, type of exposure (e.g., daily gavage versus *ad libitum* diet). In considering NAM data compared with in vivo data, the universe of considerations and questions expands considerably and includes: Cell culture or cell free system? Cell type (e.g., primary versus immortalized; human versus nonhuman)? Is metabolism involved in the assay system? Genes or proteins? Empirical observation or computational prediction? Thus, trying to compare, infer, interpolate, or extrapolate NAM concentration-response information to phenotypic outcome data derived from whole animals or humans is inherently difficult, even with the advent of in vitro-to-in vivo extrapolation (i.e., reverse toxicokinetics) approaches. Comparative evaluations of dose additivity between different levels of biological organization may be informed not so much by single point estimates, but rather by multiple points along a dose-response continuum for each level of biological organization considered. This approach may help characterize the relative potency similarities/differences across a spectrum of concentration-response (NAM data) and dose-response (apical outcome data) points, facilitating the dose-dependent relative potency factor (RPF) approaches (e.g., harmonic mean) discussed in Section 4.

Another reasonable approach may be to identify bioactivity effect doses. Because nonapical effect information within any given pathway may be derived from molecular-, cellular-, and/or subcellular organellar-based assays, dose or concentration levels might be identified where there is, or is not, some significant level of bioactivity or perturbation, compared with controls. These bioactivity effect doses (e.g., lowest-observed-effect level [LOEL]; lowest-observed-transcriptional-effect level [LOTEL]) are conceptually comparable to lowest-observed-adverse-effect levels (LOAELs) for adverse apical effects/outcomes (Judson et al. 2011; Paul Friedman et al. 2020). Although PODs are readily attainable from NAM data,

currently there are limited data by which to evaluate uncertainties, qualitatively or quantitatively, for extrapolations from in vitro responses to in vivo outcomes. It also is unclear whether traditional uncertainty factors (UFs; e.g., animal-to-human or human interindividual variability) could be appropriate to cover the extrapolation from nonapical to apical outcome data. Therefore, until consensus methods of accounting for uncertainty in extrapolation from nonapical data are available, many NAM outputs require identification of candidate (nonapical) PODs, rather than a reference value (RfV), although application of default UFs to NAM-based PODs could result in RfV-like values. The implication is that for mixture components for which derivation of an RfV based on some apical endpoint is not possible, the most basic of dose-additivity approaches (e.g., hazard index [HI]) might not be applicable in a traditional context. Rather, if a POD can be identified based on nonapical (i.e., TP; KEs) data, without apical outcome, some other measure may be warranted to inform potential mixtures hazard, such as an HI that integrates NAM-based RfV-like values, a cumulative margin-of-exposure, or a POD index (Sarigiannis and Hansen 2012). The usefulness of such alternative methodologies in the context of mixtures might be envisioned for screening or prioritization applications, such as initial screening for chemicals of potential concern at Superfund sites or prioritization to move chemicals from the preliminary Drinking Water Contaminant Candidate List to the consideration of chemicals for the Drinking Water Contaminant Candidate List. Importantly, this type of NAM evaluation could potentially save resources in both risk assessment and risk management.

The full translational application of NAM data to risk assessment ideally would span the entire fit-for-purpose continuum, from basic screening and prioritization to human health assessment (Thomas et al. 2019); however, the information must be assembled in a way that allows for easy use and interpretation. The nexus between 21st century data generation and assessment application has been significantly informed by publication of frameworks or approaches to integrating diverse data streams (OECD 2013; Tollefsen et al. 2014). Some of these structured approaches, such as the Integrated Approach to Testing and Assessment, provide a practical framing for incorporation of traditional and NAM data streams into evaluations of potential health hazard(s) and concentration- or dose-response. Such structured data stream integration approaches also facilitate identification of, and provide an opportunity to fill, data gaps. Integrated frameworks promote the leveraging of diverse data platforms and information, such as systematic literature review and evidence mapping of existent chemical studies; structure-activity (e.g., read-across) evaluations that entail structural properties; physicochemical metrics; absorption, distribution, metabolism, and excretion data; and a broad array of bioactivity information (e.g., Tox21/ToxCast). In a mixture assessment context, integrated data stream approaches may significantly advance the application of NAM data in chemical similarity grouping strategies and quantitative dose-response (e.g., additivity) evaluations.

A key aspect of integrated data stream approaches is the identification of critical pathways (i.e., AOPs/MOAs) associated with a given adverse health outcome (OECD 2017). In coordination with U.S. and international partners, advancement of AOP project foci, including within U.S. EPA's Office of Research and Development, has made tremendous progress over the past few years but much work remains, particularly in identifying translational application to risk assessment (Chauhan et al. 2021; Edwards et al. 2016; Fay et al. 2018; Knapen et al. 2018; LaLone et al. 2017; Paini et al. 2022; Svingen et al. 2021; Villeneuve et al. 2014b). For example, AOPs have traditionally been posited as chemical agnostic, potentially limiting the ability to confidently assign chemicals to more refined MOA-type groupings for mixtures risk assessment purposes. Further, chemicals may belong to more than one AOP grouping or classification. Some AOPs are virtually independent of one another, while others form interconnected networks leading to common health outcome(s) (i.e., at least one or more shared KEs in more than one AOP). Although the challenges associated with interpreting NAM/AOP data may be overcome in the long term, in the near term, leveraging other strengths of the various platforms could potentially make hazard and dose-response assessment decisions more efficient for the multitude of chemicals considered in a mixtures risk context. In addition to structure, physicochemical, and toxicokinetic information from NAM platforms (e.g., read-across; quantitative structure-activity relationship), potential adverse health outcomes of chemical mixtures may be informed using an integrated data stream approach that includes AOP characterization in a way that facilitates decisions on qualitative "hazard" groupings (i.e., more "MOA-like") and associated dose-response assessment when AO data derived from traditional bioassays are lacking.

One such characterization approach might be AOP "footprinting." The overarching principal of AOP footprinting is the stepwise profiling and comparison of AOPs at the level of KEs moving backward from the most downstream KE to the MIEs [Figure 5-2; for further details see Lambert (2022)].

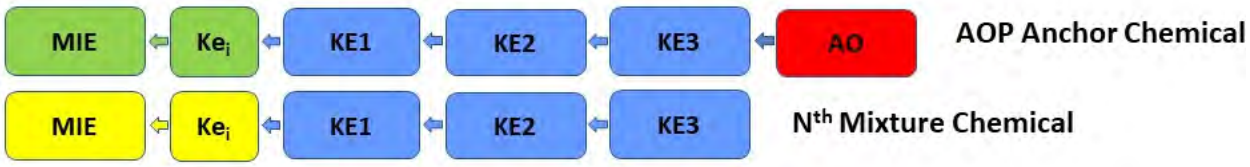


Figure 5-2. General adverse outcome pathway footprinting concept.

AOP data for mixture components are compared/contrasted with an anchor chemical for that AOP grouping, in retrograde fashion from AO to MIE to identify the most downstream common KE. [Adapted from Lambert (2022)]. AO = adverse outcome; AOP = adverse outcome pathway; KE = key event; Ke_i = one or more upstream biological events that may differ between an AOP anchor chemical and other AOP group members; MIE = molecular initiating event.

This approach may help identify commonality among mixture chemicals at a level of biological organization that is more functionally relevant to a health outcome (e.g., significantly

decreased androgen commonly leads to increased incidence of epididymal and testicular effects in male offspring). Footprinting might be less helpful for those chemicals that lack KE information near the apical outcome (e.g., lack of androgen level information [late KE] but evidence of decreased CYP450c17 activity [early event in just one pathway leading to testosterone formation]). The general steps of the approach are (1) to identify all AOPs suspected of causality in a given health outcome; (2) for each AOP, identify candidate anchor or index chemicals that have the most replete biological databases (ideally spanning events from the MIE to the AO); (3) identify the most downstream KE(s) within a given AOP for an index or anchor chemical along with the WOE supporting a causal relationship to a given AO (i.e., the AO either will not occur without said KE or at least the incidence and/or magnitude of the outcome is significantly diminished without it)—this KE is the "footprint" for a given AOP, and for any mixture, the objective is to identify a footprint for AOPs suspected of being active in a given health outcome; (4) mixture chemicals are then evaluated for KE footprint(s) and assigned to the appropriate "footprint" category based on WOE for similarity to the AOP anchor/index chemical; and (5) the key footprint event concentration-response relationships for every chemical within a footprint category are then used to evaluate mixture additivity for that AOP via one or more component-based approaches (e.g., RPF; HI). When AO data are lacking, the integration of AOP footprinting with structural, physicochemical, and toxicokinetic information may significantly advance the application of NAM data into mixtures risk assessment. For example, integrated data stream approaches might provide a WOE hazard categorization or grouping strategy, as well as a critical alternative for dose-response/additivity assessment of environmental chemicals.

In summary, NAM toxicity approaches have the capacity, far beyond traditional toxicity bioassays, to test large numbers of chemical mixtures. Such testing could include evaluating various mixing ratios of key components as well as testing at low doses. Such testing could also compare mixtures that had been isolated or concentrated from different sources. Additionally, NAM assay data, coupled with chemo- and bioinformatics, exposure, and toxicokinetic information, have the potential to allow intelligent development of groupings for chemicals for evaluating hazard and dose-response in a component-based mixtures context. The nature of these analyses suggests the need for a qualitative analysis of uncertainties. Efforts to develop quantitative uncertainty methods for NAMs are ongoing (Eccles et al. 2023; Escher et al. 2022) but need to be expanded to address complexities in mixture risk assessment applications.

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APPENDIX A. METHODS FOR EVALUATING CONSTANT RELATIVE POTENCY

This appendix addresses the determination of mixture component dose-response curves (DRCs) with similar shapes using the U.S. Environmental Protection Agency (U.S. EPA) Benchmark Dose Software (BMDS, v3.3) as an example platform (U.S. EPA 2022a). In addition, it expands on the topics covered in Sections 3 and 4 and provides additional examples of some of the methods described therein. This appendix does not address issues related to departures from additivity, such as interactions among components. There is an assumption that similarity of toxic action has already been determined to hold for the chemicals of concern. This does not exclude the possibility of differently shaped component DRCs. Section A.1 presents a statistical method to determine for dichotomous endpoints whether mixture components have differently shaped DRCs. Section A.2 then, also for dichotomous endpoints, expands on the material presented in Section 4 relating to the evaluation of relative potencies (RePs) of mixture components when DRC shapes are the same. Section A.3 further discusses methods to evaluate mixture risk for dichotomous endpoints when component DRC shapes are not the same.

Text Box A-1

ReP_x Functions

Figure A-1 is an example of the ReP_x function for the dichotomous Weibull model in Table A-3. Figure A-1 shows how ReP varies with dose for two different scenarios. The nominal RPFs are determined at the Weibull scale parameter (x = 63.2) and are set to 1 for convenience.

- *Scenario A compares two dose-response functions with Weibull powers of 2 and 1 for the index and comparison chemicals, respectively. Figure A-1 (A) shows that the ReP increases to 100 at a response level of 10^{-4} (x = 0.01) but is less than 5 within the lower range of the experimental data (x = 5).*
- *For Scenario B, the difference in the Weibull powers increases threefold, but the order is reversed; the index chemical has the lower power. In Figure A-1 (B) the ReP decreases by almost three orders of magnitude from the nominal RPF at a response level of 10^{-4} and is about fivefold less than the nominal RPF within the lower range of the experimental data.*

A.1. TESTING FOR LACK OF CONSTANT RELATIVE POTENCY WHEN SHAPE PARAMETERS ARE DIFFERENT

Chen et al. (2001) used a likelihood-ratio test to evaluate the equivalence of "slopes" (in their generalized dose-response model), which is equivalent to testing for lack of constant relative potency. They presented examples showing application of the method in grouping chemicals by similar slope and evaluating the dose-additive risk for different exposure levels (Chen et al. 2001; Chen et al. 2003). This approach, however, requires programmable statistical-modeling software, such as R® (R® Core Team 2023) or SAS® (SAS Institute Inc. 2013) and thus is not practicable in U.S. EPA's BMDS (v3).

Alternatively, the standard errors of the parameter values provided in the BMDS output can be used to determine whether two parameter values are unlikely to be equal. Confidence intervals for the parameter values are reported by BMDS, which allow for comparison of the maximum-likelihood estimates (MLEs) of two shape parameter values. If the confidence intervals do not overlap, the MLE values can be considered different.[50] An important consideration in using the confidence intervals reported by BMDS is that they assume a normal distribution of the errors, which might not be a good approximation in some cases. In particular, if the parameter value must be greater than zero (e.g., power or slope parameters) and the standard error is large, the *p*-values will be substantially underestimated near the level of significance.[51] In these cases, an alternative approach is to calculate the confidence interval in log space; this is accomplished by estimating the standard error of the logarithm of the parameter, $se_{\log}$. The corresponding confidence interval is computed by $\theta \pm z_\alpha \times se_{\log}$, where z_α is the unit normal distribution *z*-score for significance level α ($\alpha = 0.025$ for a 95% confidence interval). The relative performance of the two confidence interval alternatives has not been evaluated.

Of particular interest would be the rejection of parameter equivalence when, in fact, the two parameters are not different ("false positive"). For larger proportional standard errors (approximately 40% of the largest MLE or larger), the $se_{\log}$ confidence interval is probably more accurate. However, for very large standard errors, neither approach is likely to be highly discriminating, as very large differences in the parameter values would be necessary to achieve statistical significance. A statistically significant difference in shape parameter values does not mean that dose additivity at some level of biological organization is ruled out, as discussed in Sections 3 and 4. Statistical significance could also be obtained with nonrandom errors in the data. Nonrandom errors, particularly in the lowest and highest treatment-dose groups will tend to

[50]However, overlapping confidence intervals do not necessarily indicate lack of statistical significance. (Source: https://cscu.cornell.edu/wp-content/uploads/ci.pdf).

[51]If $se > 0.51$, the lower bound of the 95% confidence interval will be negative, even for power and slope parameters.

produce differences in the shapes when they are actually the same (Slob and Setzer 2014). Assessment of nonrandom errors requires more information than is usually available in a typical animal bioassay and is not addressed further in this document. Finally, the standard errors generated in BMDS are approximate, as will be the confidence intervals derived from them. A determination of parameter value differences logically would include the magnitude of the differences, as well as the relationship of the confidence intervals.

A practical limitation of this method is that BMDS often fails to generate standard errors for fitted parameter values, even when the model has converged. This behavior may be due to unusual sensitivity of the variance-covariance algorithm to starting values, as found for a similar algorithm by Moerbeek et al. (2004). More consistent and accurate standard errors can be obtained by more computationally intensive routines such as the likelihood ratio and bootstrap methods (Crump and Howe 1985; Moerbeek et al. 2004). These routines require computing platforms other than BMDS but can be coded using other statistical software.

A.2. EVALUATING RELATIVE POTENCIES WHEN DOSE-RESPONSE SHAPES ARE THE SAME (DICHOTOMOUS MODELS)

If the fitted shape parameters for the compounds and endpoint under consideration are determined to be the same (e.g., no significant differences have been found and constant relative potency holds), the assessor can compute relative potency factors (RPFs) that apply to any exposure level. The first step is to estimate the common shape parameter.

If, among the fitted shape parameters for the compounds and endpoint under consideration, there is no reason to choose one as the true shape, each can be considered as an independent estimate of the "true" (common) shape. In this case, the common shape is best estimated by simultaneously fitting the distribution parameters and RPFs to the combined data. Chen et al. (2001) presented a generalized dose-response model for combined exposures and demonstrated its application for several compounds using the log-logistic, dose-response model. Again, however, a simultaneous-fitting approach is not possible in BMDS.

As a simplification for BMDS users, the common shape can be estimated as a function of the individual estimates. Olmstead and LeBlanc (2005), for example, used an average power for three cholinesterase inhibitors as representative of that common mode-of-action group. For uneven mixture proportions, a weighting of the individual shape parameters by their mixture proportions could be considered. After estimating the common shape parameter value, the dose-response model can be refitted to each data set, with the shape parameter value fixed at that estimate (that is, specified in BMDS); this step can probably be skipped in most cases when the

shape parameter values are similar. Shape-normalized RePs[52] can then be estimated as in Table A-1 or Table A-2 using any of the compounds as the index chemical.

An example of the foregoing procedure for a hypothetical scenario of three compounds is presented in Table A-3 and Table A-4. The hypothetical dichotomous data sets (see Table A-3) are given in arbitrary dose units for five dose groups, with responses for an unspecified identical endpoint shown as number of responders out of 50 animals per dose group. The fitted parameter values and fitted model *p*-value (BMDS Test 4) are given for the unconstrained BMDS Weibull model fit to the individual component data (the "native" model). For the constrained fit, the values are obtained by fixing the power parameter at the average value across all three compounds (the "averaging" model). In addition, the Weibull model parameters and RePs were fit simultaneously to the combined data (the "complete" model) using the procedure of Chen et al. (2001), modified for the Weibull function;[53] the modeling results are given in Table A-4 (for the "complete" model). No statistically significant differences were found in the power parameter values for either the native or complete model fits, so assuming a common shape is reasonable. The ReP values are given for each fit, with Compound 1 chosen as the index chemical. The RePs from the complete model are considered the best estimate of the true RePs. The RePs from the averaging model differ from those of the complete model by only 1%, which is a modest improvement from the native fit with ReP differences of 10–30%. The performance of the averaging model for more compounds and across varying parameter ranges has not yet been evaluated in the literature, so the degree of accuracy for different scenarios cannot be predicted.

[52]In this context "ReP" is distinguished from "RPF" as a more general term, implying ad hoc computation. "RPF" is reserved for the more formal context of established RPFs.

[53]The fitted model is $\Pr(d_1, d_2, d_3) = b + (1-b)\,F(d_1k_1 + d_2k_2 + d_3k_3, \alpha, \lambda)$, where $\Pr(d_1, d_2, d_3)$ is the probability of a response given a specific dose of each of the three compounds (d_1, d_2, d_3), b is the background response at zero dose, F is the Weibull function in location (α)/dispersion (λ) format [as in R® or S-Plus®], k_1, k_2, and k_3 are the respective RePs for the three compounds, any of which is set to 1 (index compound). The model is fit to the combined data for all three compounds.

Table A-1. Constant Relative Potency Equivalence Definitions and Relative Potency Functions for Benchmark Dose Software Dichotomous Dose-Response Functions

Function	Mathematical form[a]: prob (response) = $p(x,\theta)$	BMDS parameter names	Parameter reformulation[b]	Constant relative potency equivalence (k = ReP)[c]	ReP$_x$ generation function[d]
Logistic[e]	$\left(1+e^{-(\alpha+\beta x)}\right)^{-1}$	α = "intercept" β = "slope"	None	$\alpha_2 = \alpha_1$ $k = \beta_2/\beta_1$	$\frac{[\text{logit}(p) - \alpha_1]\beta_1^{-1}}{[\text{logit}(p) - \alpha_2]\beta_2^{-1}}$
Probit[f]	$\Phi(\alpha + \beta x)$	α = "intercept" β = "slope"	None	$\alpha_2 = \alpha_1$ $k = \beta_2/\beta_1$	$\frac{\Phi^{-1}(p, \alpha_1, \beta_1)}{\Phi^{-1}(p, \alpha_2, \beta_2)}$
Gamma[g]	$\frac{1}{\Gamma(\alpha)}\int_0^{\beta x} t^{\alpha-1}\ e^{-t}dt$	α = "power" β = "slope"	None	$\alpha_2 = \alpha_1$ $k = \beta_2/\beta_1$	$\frac{\Gamma^{-1}(p, \alpha_1, \beta_1)}{\Gamma^{-1}(p, \alpha_2, \beta_2)}$
Log-logistic[h]	$\left(1+e^{-(\alpha+\beta \ln x)}\right)^{-1}$	α = "intercept" β = "slope"	η ("ED$_{50}$") = $e^{-\alpha/\beta}$	$\beta_2 = \beta_1$ $k = \eta_1/\eta_2$	$\frac{e^{[\text{logit}(p)-\alpha_1]\beta_1^{-1}}}{e^{[\text{logit}(p)-\alpha_2]\beta_2^{-1}}}$
Log-probit[i]	$\Phi(\alpha+\beta \log x)$	α = "intercept" β = "slope"	μ ("GM") = $e^{-\alpha/\beta}$	$\beta_2 = \beta_1$ $k = \mu_1/\mu_2$	$\frac{e^{\Phi^{-1}(p,\alpha_1,\beta_1)}}{e^{\Phi^{-1}(p,\alpha_2,\beta_2)}}$
Weibull	$1 - e^{-\beta x^{\alpha}}$	α = "power" β = "slope"	λ ("scale") = $\beta^{-(1/\alpha)}$	$\alpha_2 = \alpha_1$ $k = \lambda_1/\lambda_2$	$\frac{\lambda_1\left(\log[(1-p)^{-1}]^{\alpha_1^{-1}}\right)}{\lambda_2\left(\log[(1-p)^{-1}]^{\alpha_2^{-1}}\right)}$
Quantal linear	$1 - e^{-\beta x^{\alpha}}$	α = "power" = 1 β = "slope"	None	Always constant $k = \beta_2/\beta_1$	None (β_2/β_1 constant)

Function	Mathematical form[a]: prob (response) = $p(x,\theta)$	BMDS parameter names	Parameter reformulation[b]	Constant relative potency equivalence (k = ReP)[c]	ReP$_x$ generation function[d]
Multistage[j] (polynomial)	$1 - e^{-\sum_{j=1}^{n} \beta_j x^j}$	dose coefficients β_j (j = 1…n)	None	$\beta_{j2} = \beta_{j1} k^j$ k = mean $\left(\frac{\beta_{j2}}{\beta_{j1}}\right)^{j^{-1}}$ averaged across all j[†]	$\frac{\text{poly}^{-1}(\log(1-\text{p}),\beta_{\text{j}_1})}{\text{poly}^{-1}(\log(1-\text{p}),\beta_{\text{j}_2})}$

[a]x = dose; θ = vector of parameters; background response parameter omitted (see text).

[b]For native BMDS parameters not scalable, mathematically equivalent parameters substituted.

[c]k defines ReP_2 relative to ReP_1.

[d]Calculation formula for determining ReP$_x$ at fractile p ($p = x$ in ED$_x$) if constant relative potency parameter values are not equal (i.e., $\alpha_2 \neq \alpha_1$, $\beta_2 \neq \beta_1$, etc.).

[e]logit(p) = log[$p \div (1-p)$].

[f]Φ is the unit normal probability function, and Φ^{-1} is the inverse unit normal probability function (quantile function), both given in terms of the probit intercept and slope. Equivalent forms for Φ and Φ^{-1} expressed in terms of the mean and standard deviation can be found in this appendix.

[g]Γ is the gamma function [$(n-1)!$]; Γ^{-1} is the inverse gamma probability function (quantile function) and must be solved numerically.

[h]Use these relationships for the dichotomous Hill function, which is mathematically identical to the log-logistic but allows for a maximum response of less than 1 (see text for discussion of background responses and maximum responses less than 1).

[i]α and β are given in terms of the logarithm of dose.

[j]poly^{-1} is the polynomial root function for calculating quantiles of the (linearized) multistage model; k is the average of the individual values calculated for each β_j.

[†]If both β_{jx} are 0 ($\beta_{j1} = \beta_{j2} = 0$), they can be omitted; k is undefined if one $\beta_{jx} = 0$ and the corresponding β_{jx} is not but can be approximated as the asymptote as $p(x,\theta) \rightarrow 1$.

Source for Columns 1–3: BMDS (U.S. EPA 2022a).

BMDS = Benchmark Dose Software; ED$_x$ = effective dose for x response; GM = geometric mean (= median); ReP = relative potency.

Table A-2. Constant Relative Potency Equivalence Definitions and Relative Potency Functions for Benchmark Dose Software Continuous Dose-Response Functions

Function	Mathematical form[a] $\mu(x) = f(x,\theta)$	BMDS parameter names	Constant relative potency equivalence[b] (k = ReP)	ReP$_x$ generation function[c]
Hill[d]	$\gamma + \frac{\nu x^n}{\kappa^n + x^n}$	γ = "intercept" ν = "sign" κ = "slope" (ED$_{50}$) n = "power"	$n_2 = n_1$ $k = \kappa_1/\kappa_2$	$\frac{e^{[\text{logit}(p)-\kappa_1]n_1^{-1}}}{e^{[\text{logit}(p)-\kappa_2]n_2^{-1}}}$
Exponential, model 2, 4[e]	$ae^{\delta bx}$ $a\left[c-(c-1)e^{\delta bx}\right]$	a = "bkg. response" δ = "sign" b = "slope" c = "asymptote"	Always constant $k = b_2/b_1$	None (b_2/b_1 constant)
Exponential, model 3, 5[f]	$ae^{\delta bx^d}$ $a\left[c-(c-1)e^{\delta bx^d}\right]$	a = "bkg. response" δ = "sign" b = "slope" d = "power" c = "asymptote"	$d_2 = d_1$ $k = b_2/b_1$ $k = b_1^{-(1/d_1)}/b_2^{-(1/d_2)}$	$\frac{\lambda_1\left(log[(1-p)^{-1}]^{d_1^{-1}}\right)}{\lambda_2\left(log[(1-p)^{-1}]^{d_2^{-1}}\right)}$
Polynomial[g]	$\sum_{j=0}^{n}\beta_j x^j$	dose coefficients β_j ($j = 0\ldots n$)	$\beta_{j2} = \beta_{j1}k^j, j > 0$ $k = \langle\left(\frac{\beta_{j2}}{\beta_{j1}}\right)^{j^{-1}}\rangle, j > 0$[h]	$\frac{\text{poly}^{-1}\left(1-p,\beta_{j_1}\right)}{\text{poly}^{-1}\left(1-p,\beta_{j_2}\right)} j > 0$
Linear	$\beta_0 + \beta_1 x$	β_0 = (intercept) β_1 = (slope)	Always constant $k = \beta 1_2/\beta 1_1$	None (β_2/β_1 constant)
Power	$\gamma + \beta x^d$	γ = "intercept" β = "slope" d = "power"	$d_2 = d_1$ $k = \beta_1^{-(1/d_1)}/\beta_2^{-(1/d_2)}$	$\frac{(p\beta_1^{-1})^{d_1^{-1}}}{(p\beta_2^{-1})^{d_2^{-1}}}$

[a] $\mu(x)$ = mean response at dose = x; θ = vector of parameters for indicated distribution.

[b] k defines ReP$_2$ relative to ReP$_1$.

[c] Calculation formula for determining ReP$_x$ at fractile p ($p = x$ in ED$_x$) if constant relative potency parameters are not equal (i.e., $\alpha_2 \neq \alpha_1$, $\beta_2 \neq \beta_1$, etc.).

[d] ν ("sign") is the asymptote for large x, but can be positive or negative depending on the direction of the change in response; the ReP$_x$ function is the same as for the dichotomous log-logistic, with $\beta = n$ (see Table A-1) because of the mathematical equivalence of the log-logistic and Hill functions when $\nu = 1$; logit(p) = log[p/(1−p)].

[e] $c = 0$ for Model 2; δ ("sign") = ±1 but can be incorporated into b depending on the direction of the change in response.

[f] $c = 0$ for Model 3.

[g] poly^{-1} is the polynomial root function for calculating quantiles of the polynomial model; k is the average of the individual values calculated for each β_j.

[h] If both β_{jx} are 0 ($\beta_{j1} = \beta_{j2} = 0$), they can be omitted; k is undefined if one chemical $\beta_{jx} = 0$ and the other chemical β_{jx} does not.

Source for Columns 1–3: BMDS (U.S. EPA 2022a).

BMDS = Benchmark Dose Software; ED = effective dose; GM = geometric mean (= median); ReP = relative potency.

Table A-3. Hypothetical Experimental Data

Compound	Dose[a]/ response[b]	Dose group 1	2	3	4	5
Compound 1	Dose	0	3	20	50	100
	Response	0/50	2/50	8/50	16/50	32/50
Compound 2	Dose	0	20	50	100	200
	Response	2/50	3/50	5/50	7/50	22/50
Compound 3	Dose	0	30	100	300	600
	Response	1/50	2/50	5/50	15/50	32/50

[a]Exposure dose in arbitrary units.

[b]Number responding/number on test (arbitrary identical effect for all compounds).

Table A-4. Dose-Response Modeling Results for Hypothetical Scenario

Compound	Fitted model	Weibull fit Power[a]	Scale[b]	*p*-value[c]	RPF[d]
Compound 1	Native (raw data)	1.021 (± 0.1856)	107	0.763	1
	Average power[e]	1.5035	95.6	0.293	1
	Complete[f]	1.366[g]	99.4	0.869	1
Compound 2	Native	1.438 (± 0.3484)[h]	611	0.919	0.175
	Average power	1.5035	604	0.977	0.158
	Complete	1.366	--	--	0.159
Compound 3	Native	2.052 (± 0.9284)[h]	277	0.729	0.387
	Average power	1.5035	325	0.768	0.294
	Complete	1.366	--	--	0.297

[a]α for Weibull function in Table A-1; BMDS standard errors given in parentheses where relevant.

[b]λ for Weibull function in Table A-1.

[c]Goodness-of-fit *p*-value (BMDS Test 4).

[d]$\text{Scale}_{\text{cpd.1}} \div \text{scale}_{\text{cpd.}i}$ (*k*; see Table A-1); Compound 1 selected as the index chemical.

[e]Weibull model refit to data with power parameter value fixed at average for all three compounds.

[f]Simultaneous fit of model parameters and component RePs using an extension of the Chen et al. (2001) model for the Weibull distribution; common power assumed; Compounds 2 and 3 doses scaled by their respective fitted RePs to coincide with Compound 1.

[g]No significant differences in component power parameter values; likelihood ratio test ($p = 0.335$).

[h]No significant difference from Compound 1 by comparing lognormal confidence intervals.

BMDS = Benchmark Dose Software; RPF = relative potency factor.

A.3. PREDICTING RESPONSE TO THE MIXTURE WHEN DOSE-RESPONSE SHAPES ARE DIFFERENT (DICHOTOMOUS MODELS)

A.3.1. DOSE DEPENDENCY OF RELATIVE POTENCY FACTORS

If the fitted shape parameters for the compounds and endpoint under consideration are determined to be different but the chemicals are still believed to act via the same toxicological pathway and to be dose-additive at a key step, an RPF approach may not be appropriate because the response to the mixture will depend on the choice of the index chemical (see Section 4.1.3). Text Box A-1 illustrates the behavior of variable ReP_x functions for a hypothetical scenario, showing the rapid increase or decrease of relative potency at low exposure levels depending on the choice of index chemical (see Figure A-1).

The assessor, however, can still estimate the potential range of response to the mixture using the alternative approaches described in Section 4.1.3. The assessor could compute an ReP_x using BMDS, as described later in this section, or use the ReP_x functions in Table A-1 and Table A-2[54] to calculate an appropriate ReP for each specific exposure value. However, as discussed in Section 4.1.3, this approach is subject to the issue of dependence of the predicted response to the mixture on the choice of index chemical. The ReP_x functions are also error functions if the parameter differences are false positives, so the potential for error can be assessed if there is uncertainty about the statistical significance of the difference in shapes.

When the predicted mixture risk depends on the index chemical selected for ReP_x models, there are several alternative approaches (presented in Section 4.1.3) for approximating mixture risk. These approaches are the harmonic mean method, the use of biologically based models and approximate limits of the predicted mixture response, and the restriction of modeling to the low-response portion of the DRC.

[54]Table A-1 and Table A-2 include the ReP_x functions, which are simplified versions of the ratios of the quantile functions for the two mixture components, independent of the absolute value of the location parameters.

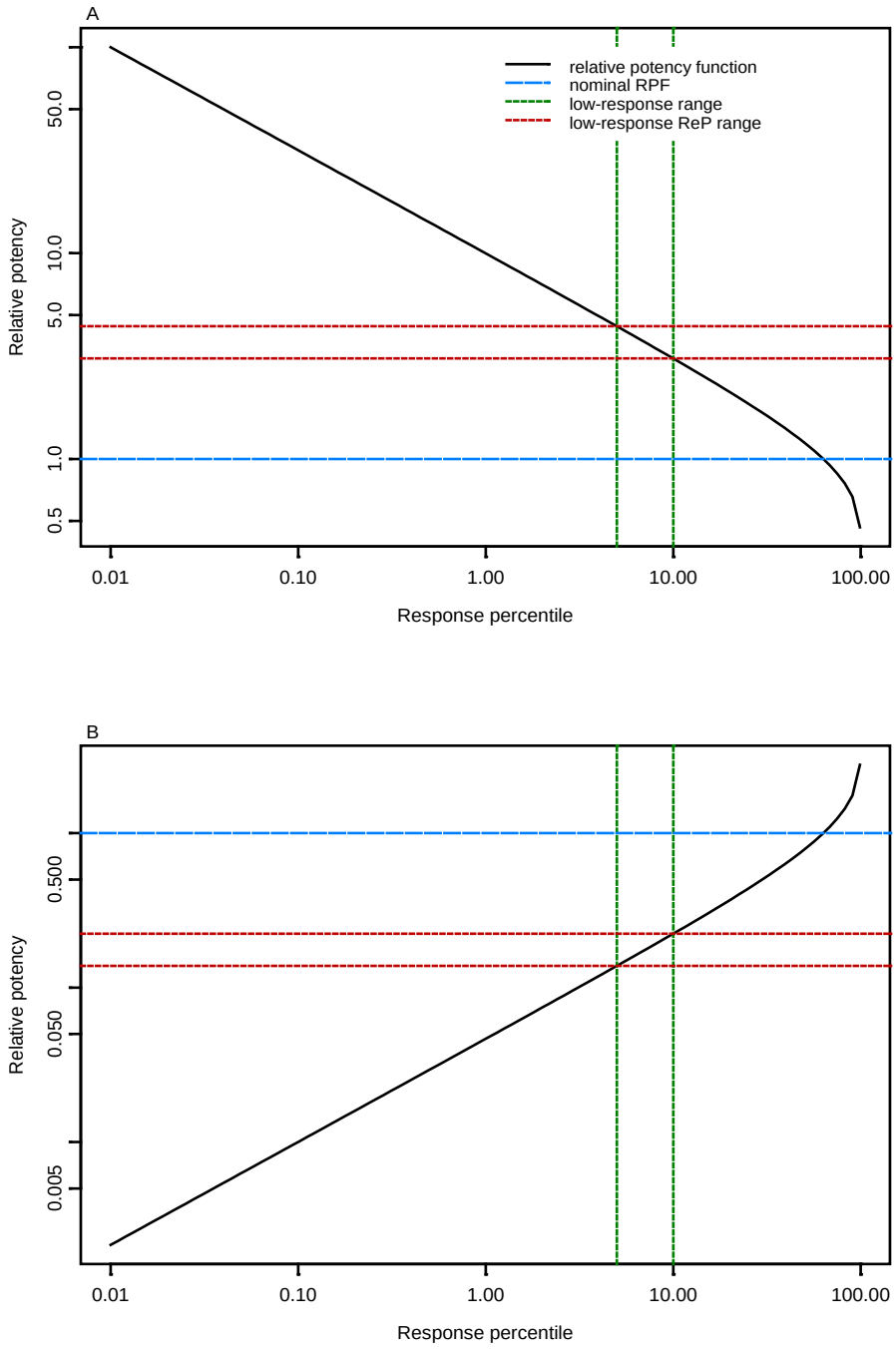

Figure A-1. Dose-dependent relative potency plots illustrated with the Weibull dichotomous dose-response function.

The relative potency function is $ED_{x[1]} \div ED_{x[2]}$. (A) WP for Chemical 1 = 2; WP for Chemical 2 = 1. (B) WP for Chemical 1 = 1; WP for Chemical 2 = 3. The nominal RPF would apply under constant relative potency. The low-response range is set at 5–10% to represent typical animal bioassays.
ED_x = effective dose; RPF = relative potency factor; WP = Weibull power.

A.3.2. THE HARMONIC MEAN METHOD

The harmonic mean formula is a transformation of the isobole formula first published by Loewe and Muischnek (1926). The isobole formula states the mathematical relationship between a mixture dose and individual doses of the mixture components producing the same response (see Eq A-1). The formula is essentially a test for additivity, where a value of 1 indicates dose (or concentration) additivity. If the equation satisfies the equality (i.e., = 1), a plot of the dose of one chemical against another for all combinations of two chemicals producing the same specified effect in a mixture will be a straight line. Berenbaum is perhaps better known for his expansion of the isobole concept, with the isobole equation frequently referred to as "Berenbaum's equation" (Berenbaum 1981; 1985). The "harmonic mean"[55] transformation of the isobole formula converts it from a test for additivity to a predictive model for a total mixture dose (with fixed component ratios) associated with a specified response level (see Eq A-5)[56] and was first identified by Smyth et al. (1969) who used it to evaluate responses to mixtures of pesticides at the ED_{50}. The method has been used extensively since 2000 as a predictor of entire dose-response functions for mixtures (see Section 4.1.3.2). The first complete derivation of the harmonic mean formula appears to be in a book chapter (Svendsgaard and Hertzberg 1994); adapting the notation from that publication slightly, the derivation of the harmonic mean equation from the isobole formula is as follows:

Start with the isobole formula;

$$\sum_{i=1}^{n} \frac{d_i}{EDx_i} = 1 \qquad \text{(A-1)}$$

divide through by the total mixture dose associated with effect level x;

$$\sum_{i=1}^{n} \frac{d_i/D_{mix}}{EDx_i} = \frac{1}{D_{mix}} \qquad \text{(A-2)}$$

substitute the component proportion notation (π_i) for the fractional representation;

$$\sum_{i=1}^{n} \frac{\pi_i}{EDx_i} = \frac{1}{D_{mix}} \qquad \text{(A-3)}$$

substitute explicit component dose-response functions [$g_i(x,\theta_i)$] for the ED_{xi}; and

[55]Despite extensive use of the harmonic mean method, no formal name has been assigned to it for mixture dose-response modeling. Smyth et al. (1969) and Kortenkamp et al. (2012) mentioned the harmonic mean in connection with the formula, without formally naming it. The method has also been termed the "isobole" method by Bosgra et al. (2009) or, simply (and implicitly), "a definition of dose additivity" [Moser et al. (2005) citing Berenbaum (1985)]. The term "harmonic mean" method is used here to avoid confusion with the actual isobole formula of Loewe and Muischnek (1926).

[56]Note that each of the equations A-1 through A-4 is in the mathematical form of a harmonic mean, which is the reciprocal of the average of the reciprocals of the individual doses.

$$\sum \frac{\pi_i}{g_i(x,\theta_i)} = \frac{1}{D_{mix}} \quad \text{(A-4)}$$

invert the equation to yield the predicted total mixture dose corresponding to the mixture response, x;

$$Dx_{mix} = \left(\sum \frac{\pi_i}{g_i(x,\theta_i)}\right)^{-1} \quad \text{(A-5)}$$

where:

n = the number of chemicals in the mixture,

d_i = the dose of chemical i in the mixture,

ED_{xi} = the dose of chemical i, alone, corresponding to the mixture response, x,

D_{mix} = the total mixture dose (sum of the component doses or mass concentrations),

π_i = the mass-fraction of chemical i in the mixture (d_i/D_{mix}),

$g_i(x,\theta_i)$ = the dose-response quantile (inverse probability) function for chemical i, given probability of response, x, and

D_{xmix} = the predicted total mixture dose corresponding to the mixture response, x.

The harmonic mean method is carried out by constructing a vector of predicted mixture doses (D_{xmix}) corresponding to a sequence of response levels ("x" in ED_x) in the range of interest (i.e., anticipated response to the mixture), given the specified fractional component doses (π_i) in the mixture, using Eq A-5 [see Qin et al. (2011), Mwense et al. (2004), and Altenburger et al. (2000) for examples of the harmonic mean method]. The predicted response to the actual mixture of concern is then obtained by matching the actual total mixture dose (D_{mix}) (sum of component doses without adjustment for relative potency) to a specific predicted D_{xmix} and noting the corresponding x. An example of the basic process is shown in Figure A-2. The predicted mixture curve, however, is for the specified component fractions and so will be different for mixtures with different component fractions. Figure A-3 compares the results of the harmonic mean method for the same hypothetical chemicals presented in Figure A-2, but with different mixture component proportions. Note that the axes are reversed from the usual DRC plotting method. For the harmonic mean method, the response, rather than the dose, is the independent variable, with total mixture dose predicted from a given response level. In this example, the curve shapes are different, with both the three-component mixtures tending to "follow" the lower Weibull power (WP) single-component curve (Chemical 2) with decreasing response level. Note that Chemical 3 has a nominal RPF of 0.3 but becomes more toxic than the index chemical (Chemical 1) at a response level of about 25% (x = 0.25). In fact, the ReP_x for Chemical 3 will increase without bound at increasingly lower response levels. This illustrates the ReP_x extrapolation problem discussed previously. It also shows that the harmonic mean method shares

this problem to some degree, in that the predicted mixture dose is highly influenced by shape parameters that may not be relevant at low doses (below the observed response range). Therefore, with large differences in dose-response shapes for chemicals otherwise determined to act similarly, risk mixture predictions at exposures well below the range of observations would be treated as highly uncertain. Note that the RPF approach, for same-shape component DRCs, does not have this problem, as RePs are the same for all response levels. Additional examples are presented below.

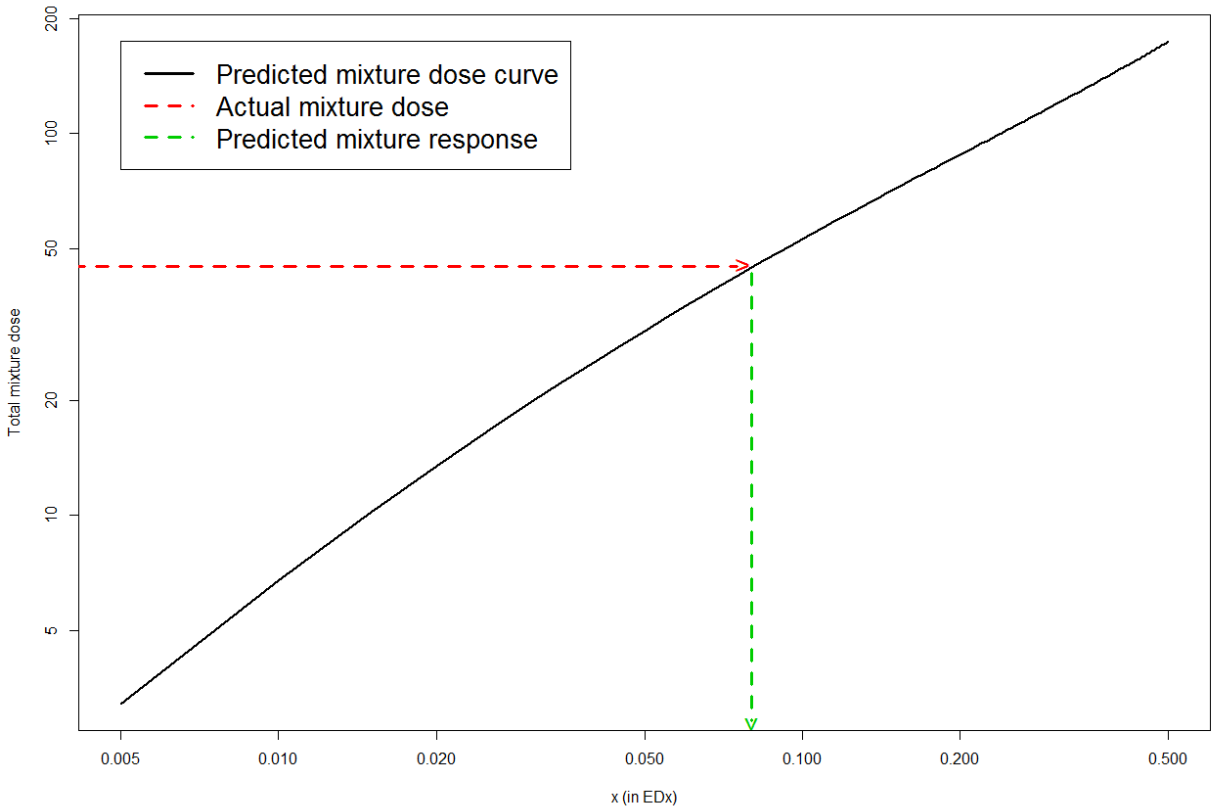

Figure A-2. Demonstration of harmonic mean method process.

Plot of hypothetical three-chemical mixture total dose (arbitrary units) prediction with Weibull dose-response functions: Chemical 1 (scale = 333, power = 2); Chemical 2 (scale = 100, power = 2); Chemical 3 (scale = 1,000, power = 0.8); actual total mixture dose = 48, with each chemical in equal mass proportions (i.e., 16 dose units each). This constructed curve holds for a fixed set of mixing ratios (i.e., with each mixture composition, a new curve is created) (see examples in appendix text).

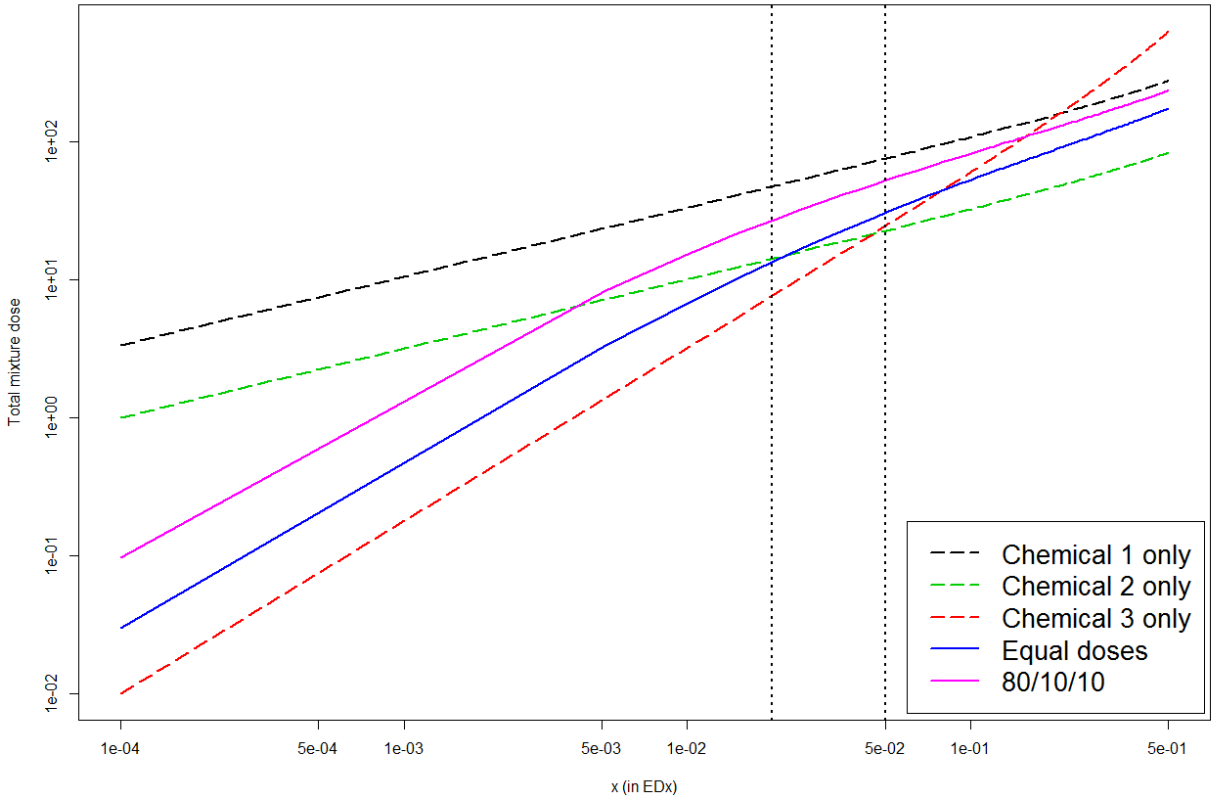

Figure A-3. Example of harmonic mean method for different mixture proportions.

Plot of same hypothetical chemicals in Figure A-2, but in different proportions. "Equal doses" corresponds to Figure A-2 scenario. "80/10/10" represents percentage mass-contributions of Chemicals 1, 2, and 3, respectively. A typical lower observed response range (2–5%) for large rodent bioassays is shown by the vertical dotted lines for comparison.

Figure A-4 to Figure A-6 provide examples of the harmonic mean method for different mixture component ratios. These figures are plots of the predicted D_{mix}, using the harmonic mean method, for hypothetical three-component mixtures for different shape-parameter combinations and different component (mass) ratios[57] in the mixture. The dose-response model parameter values are given in the figure captions. D_{mix} was computed for response probabilities ("x") ranging from 0.001 to 0.5. The results are plotted in log-log space for better visualization across the response range. D_{mix} is plotted on the y-axis because, in this case, it is the dependent variable, computed from a given response ("x"). The component mass ratios for each scenario are 100% for each chemical, only, equal doses of each chemical (33% of mass concentration each), and RPF-scaled doses, where each chemical is in equitoxic amounts relative to the nominal RPFs (calculated from the Weibull "scale" parameters, with Chemical 1 as the index chemical). A

[57]The ratios must be the same for all exposure levels.

typical lower observed response range (2–5%) for large rodent bioassays is shown by the vertical dotted lines for comparison.

Figure A-4 represents the simplest case where the component DRC shapes are all the same. It is apparent in this figure that all the curves are parallel (on a log-dose scale) when the DRC shapes are the same, which is expected for constant relative potency. Figure A-5 shows the case in which one of the component DRC shapes is different (the WP is 1/2 that of the other two chemicals). The curve shapes are different, with both three-component mixtures tending to "follow" the lower WP single-component curve (Chemical 3). Figure A-6 depicts a more extreme example of shape differences, with the WP of Chemical 3 fourfold less than that for the other two chemicals. As response level decreases, Chemical 3 quickly transforms from the least toxic component to the most toxic, with the three-component mixture predictions rapidly diverging from the other two chemicals, even in the lower observed response range.

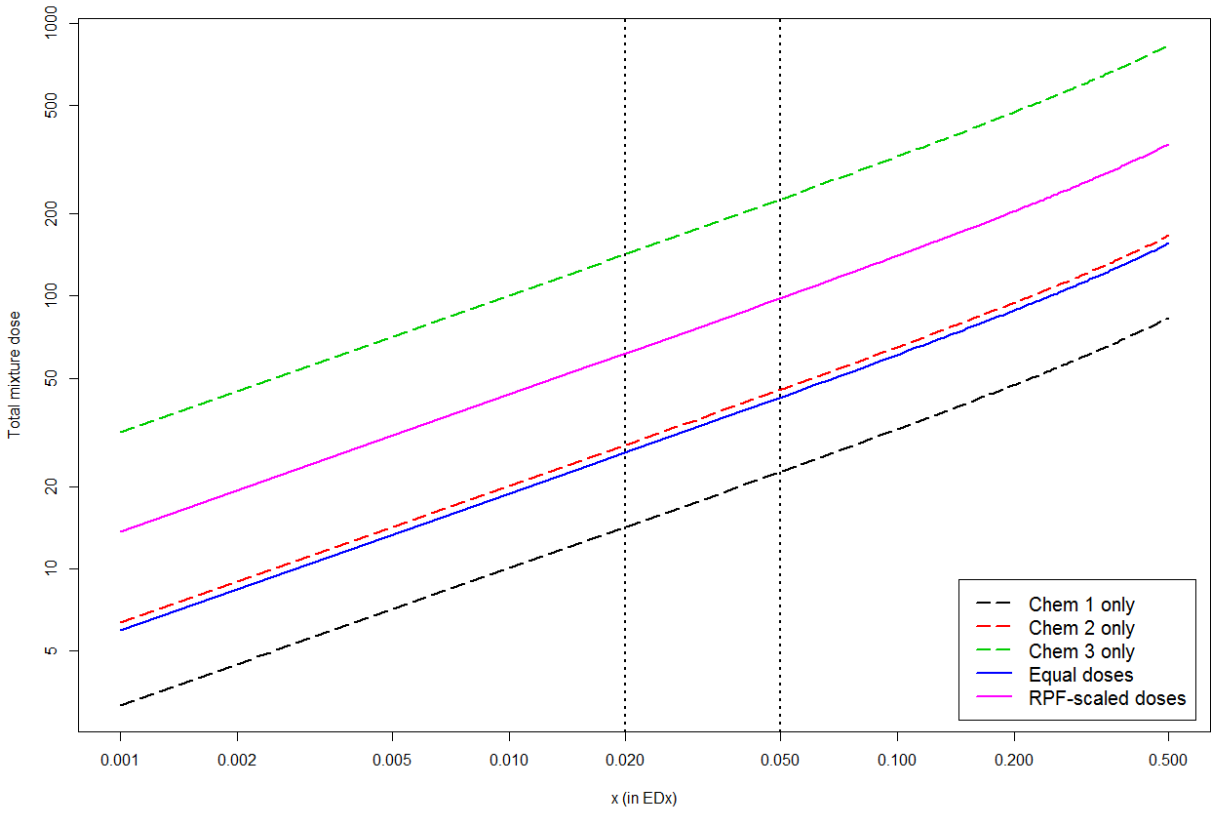

Figure A-4. Harmonic mean method demonstration I.

Shape parameters (WPs) are set at 2 for all components; nominal RPFs (computed at the Weibull scale parameter) are 1, 0.5, and 0.1 for Chemicals 1, 2, and 3, respectively; location parameter (Weibull scale) for Chemical 1 is 100 (arbitrary units).
RPF = relative potency factor; WP = Weibull power.

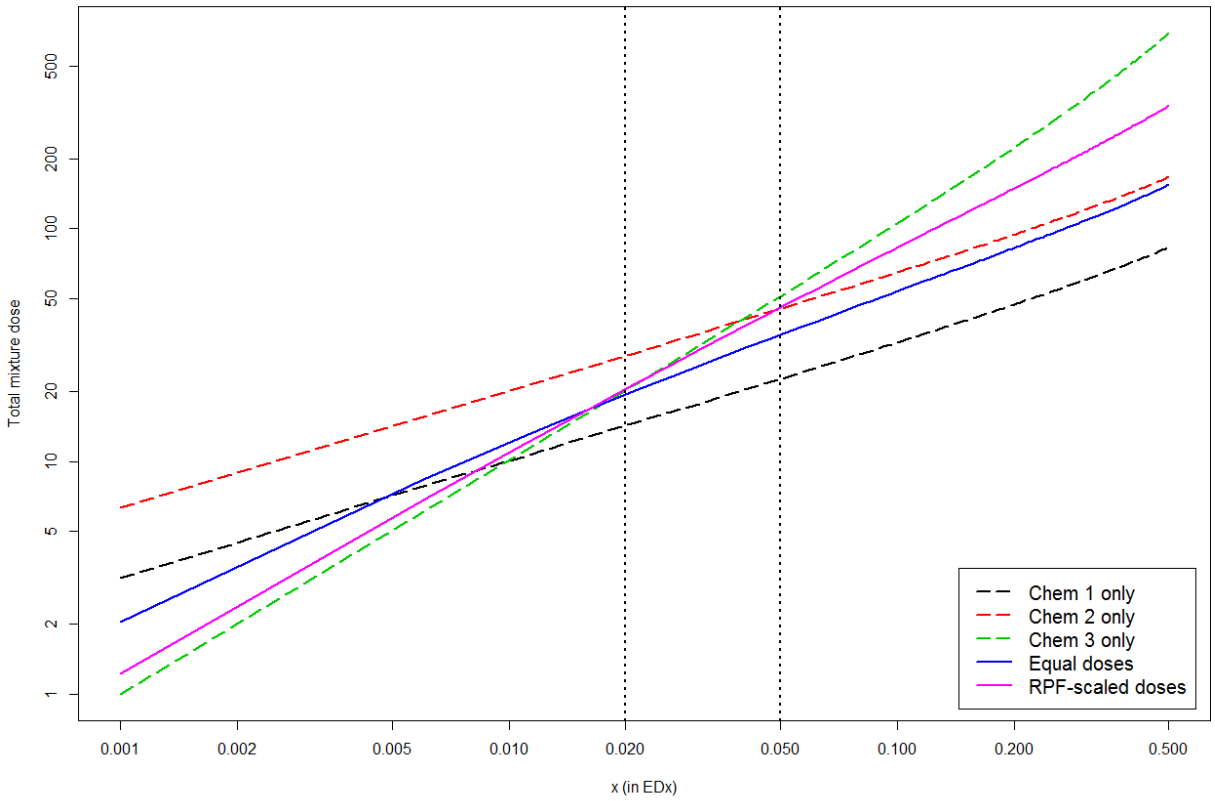

Figure A-5. Harmonic mean method demonstration II.

Shape parameters (WPs) are 2, 2, and 1 for Chemicals 1, 2, and 3, respectively; nominal RPFs are 1, 0.5, and 0.1 for Chemicals 1, 2, and 3, respectively; location parameter for Chemical 1 is 100.
RPF = relative potency factor; WP = Weibull power.

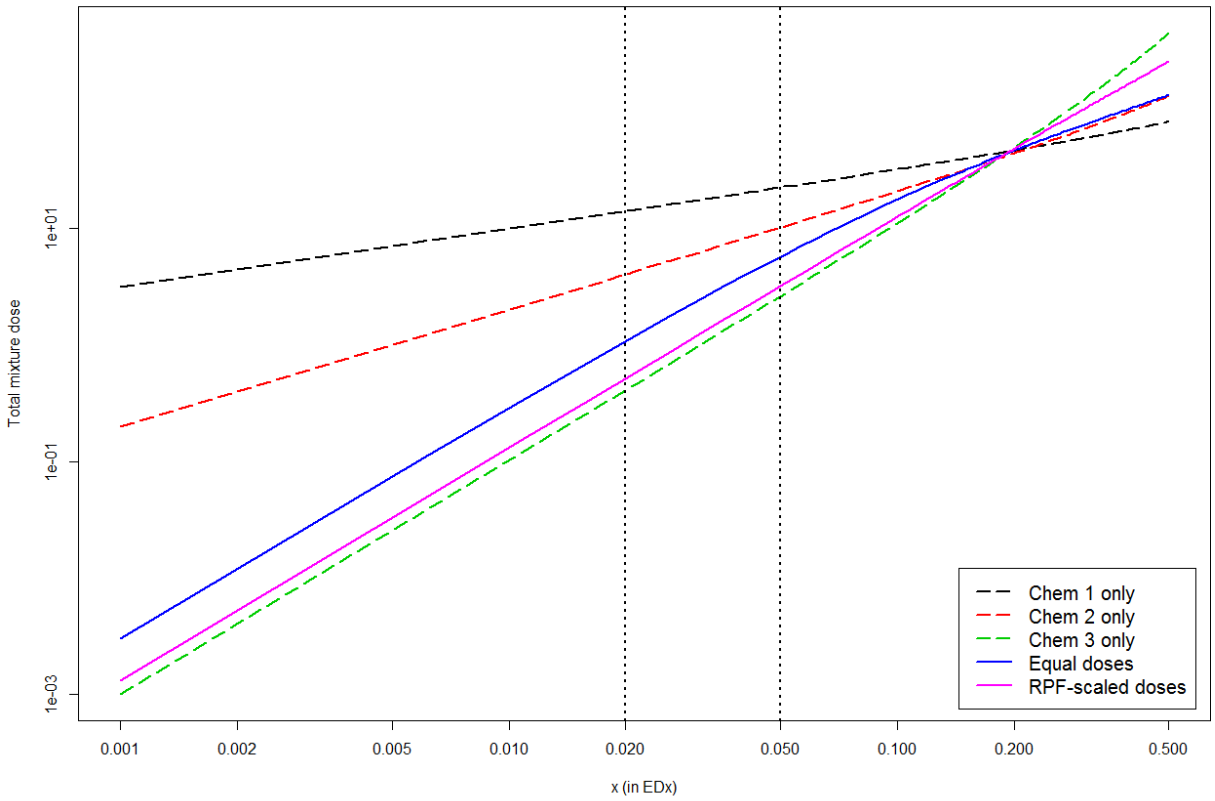

Figure A-6. Harmonic mean method demonstration III.

Shape parameters (WPs) are 2, 1, and 0.5 for Chemicals 1, 2, and 3, respectively; nominal RPFs are 1, 0.5, and 0.1 for Chemicals 1, 2, and 3, respectively; location parameter for Chemical 1 is 100.
RPF = relative potency factor; WP = Weibull power.