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# Toxicological Review of Trimethylbenzenes

[CASRNs 25551-13-7, 95-63-6, 526-73-8, and 108-67-8]

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Integrated Risk Information System
National Center for Environmental Assessment
Office of Research and Development
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# CONTENTS

# TABLES

# FIGURES

# ABBREVIATIONS

| | |
|---|---|
| AAQC | Ambient air quality criterion |
| ABR | amount of 1,2,4-TMB in the brain |
| ADME | absorption, distribution, metabolism, and excretion |
| AEGL | Acute Exposure Guideline Level |
| AIC | Akaike Information Criterion |
| ALT | alanine aminotransferase |
| ANCOVA | analysis of covariance |
| ANOVA | analysis of variance |
| AP | alkaline phosphatase |
| AST | aspartate aminotransferase |
| AUC | area under the curve |
| BAL | bronchoalveolar lavage |
| BMCL | lower confidence limit on the benchmark concentration |
| BMD | benchmark dose |
| BMDL | lower confidence limit on the benchmark dose |
| BMDS | benchmark dose software |
| BMR | benchmark response |
| BrdU | 5-bromo-2'-deoxyuridine |
| BUN | blood urea nitrogen |
| BW | body weight |
| CAAC | Chemical Assessment and Advisory Committee |
| CASRN | Chemical Abstracts Service Registry Number |
| CE | cloning efficiency |
| CHO | Chinese hamster ovary |
| CI | confidence interval |
| CMIX | average of arterial and venous blood concentrations |
| CNS | central nervous system |
| CV | concentration in venous blood |
| CVS | concentration in venous blood exiting slowly perfused tissues |
| CXEQ | concentration in exhaled breath |
| CYP450 | cytochrome P450 |
| DAF | dosimetric adjustment factor |
| df | degree of freedom |
| DMBA | dimethylbenzoic acid |
| DMHA | dimethylhippuric acid |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| $EC_{50}$ | half maximal effective concentration |
| EEG | electroencephalogram |
| EPA | U.S. Environmental Protection Agency |
| fMRI | functional magnetic resonance imaging |
| GABA | gamma-aminobutyric acid |
| GD | gestational day |
| GGT | gamma-glutamyl transpeptidase |
| Hb/g-A | animal blood:gas partition coefficient |
| Hb/g-H | human blood:gas partition coefficient |
| HEC | human equivalent concentration |
| HED | human equivalent dose |
| HERO | Health and Environmental Research Online |
| HFAN | High-Flash Aromatic Naphtha |
| HLVOC | highly lipophilic volatile organic chemical |
| HSDB | Hazardous Substances Data Bank |
| IL-8 | interleukin-8 |
| i.p. | intraperitoneal |
| IRIS | Integrated Risk Information System |
| JP-8 | jet propulsion fuel 8 |
| KCCT | kaolin-cephalin coagulation time |
| $K_m$ | Michaelis-Menten constant |
| LLF | log-likelihood function |
| LOAEL | lowest-observed-adverse-effect level |
| MCH | mean corpuscular hemoglobin |
| MCHC | mean corpuscular hemoglobin concentration |
| MCV | mean cell volume |
| MMS | methyl methanesulfate |
| MOE | Ministry of the Environment |
| NIOSH | National Institute for Occupational Safety and Health |
| NLE | neutral lipid equivalent |
| NLM | National Library of Medicine |
| NMDA | N-methyl-D-aspartate |
| NOAEL | no-observed-adverse-effect level |
| NOEL | no-observed-effect level |
| NRC | National Research Council |
| NSC | normalized sensitivity coefficient |
| OSHA | Occupational Safety and Health Administration |
| *p*-value | probability value |
| PBPK | physiologically based pharmacokinetic (model) |
| PCV | packed cell volume |
| pg | picogram |
| PMR | proportional mortality ratio |
| PND | postnatal day |
| POD | point of departure |
| $POD_{ADJ}$ | duration-adjusted POD |
| ppm | parts per million |
| QPC | alveolar ventilation rate |
| OR | odds ratio |
| QRTOTC | sum of fractional flows to rapidly perfused tissues, liver, and brain |
| QSTOTC | sum of fractional flows to slowly perfused tissues |

| | |
|---|---|
| RBC | red blood cell |
| RD | relative deviation |
| $RD_{50}$ | 50% respiratory rate decrease |
| REL | recommended exposure limit |
| RfC | reference concentration |
| RfD | reference dose |
| ROS | reactive oxygen species |
| SAB | Science Advisory Board |
| SCE | sister chromatid exchange |
| SCI | Science Citation Index |
| SD | standard deviation |
| SDH | sorbitol dehydrogenase |
| SE | standard error |
| SMR | standardized mortality ratio |
| SOA | secondary organic aerosol |
| SVEP | short-latency visual evoked potential |
| SWD | spike-wave discharge |
| TLV | threshold limit value |
| TMB | trimethylbenzene |
| TOXLINE | Toxicology Literature Online |
| TWA | time-weighted average |
| UF | uncertainty factor |
| $UF_A$ | interspecies uncertainty factor |
| $UF_H$ | intraspecies uncertainty factor |
| $UF_S$ | subchronic-to-chronic uncertainty factor |
| $UF_L$ | LOAEL-to-NOAEL uncertainty factor |
| $UF_D$ | database deficiency uncertainty factor |
| VEP | visual evoked potential |
| $V_{max}$ | ½ maximal enzyme rate |
| VOC | volatile organic compound |
| W | watt |
| WBC | white blood cell |
| WOS | Web of Science |
| $\chi^2$ | chi-squared |

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This assessment was provided for review to other federal agencies and the Executive Office of the President (EOP). A summary and EPA's disposition of major comments from other federal agencies and EOP is available on the IRIS Website. Comments were submitted by:

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DHHS/National Institute for Occupational Safety and Health
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This assessment was released for public comment on June 26th, 2012 and comments were due on August 28th, 2012. The public comments are available on Regulations.gov. A summary and EPA's disposition of the comments from the public is available in the external peer review draft assessment on the IRIS website. Comments were received from the following entities:

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Region 8, Denver, CO
Region 2, New York City, NY
Office of the Administrator/Office of Children's Health Protection
Office of Land and Emergency Management
Office of Policy

Department of Defense
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Small Business Administration/Office of Advocacy

# PREFACE

This Toxicological Review critically reviews the publicly available studies on the three isomers of trimethylbenzene (TMBs) (i.e., 1,2,3-TMB, 1,2,4-TMB, and 1,3,5-TMB) in order to identify their adverse health effects and to characterize exposure-response relationships. Because more types of studies are available for the 1,2,4-TMB isomer, it generally appears first when the individual isomers are listed. This assessment was prepared under the auspices of the U.S. Environmental Protection Agency (EPA) Integrated Risk Information System (IRIS) program.

This assessment was prepared because of the presence of TMBs at Superfund sites. Of sites on EPA's National Priorities List that report TMB isomer contamination (38 sites), 93% report 1,3,5-TMB contamination, 85% report 1,2,4-TMB contamination, 12% report 1,2,3-TMB contamination, and 17% report contamination by unspecified TMB isomers.

The *Toxicological Review of Trimethylbenzenes* is a new assessment; there is no previous entry on the IRIS Database for 1,2,3-TMB, 1,2,4-TMB, or 1,3,5-TMB. This assessment reviews information on all health effects by all exposure routes.

This assessment was conducted in accordance with EPA guidance, which is cited and summarized in the Preamble to IRIS Toxicological Reviews. The findings of this assessment and related documents produced during its development are available on the IRIS website (http://www.epa.gov/iris). Appendices for toxicokinetic information, summaries of toxicity studies, and other supporting materials are provided as supplemental information to this assessment (see Appendices C and D).

The IRIS Program released this assessment for public comment and peer review in June 2012, as it was beginning to implement systematic review. The approach to implementation is to use procedures and tools available at the time, without holding assessments until new methods become available. Accordingly, the IRIS Program edited this assessment to increase transparency and clarity and to use more tables and figures. It conducted literature searches and evaluated studies using tools and documentation standards then available. However, this assessment does differ in some its methods compared to those outlined in the Preamble, due to the phased implementation of systematic review. Notably, problem formulation materials and protocol development (Preamble, Section 3) began with assessments started in 2015, after this assessment was well into peer review. Additionally, this assessment does not fully implement the methods outlined in Section 4 of the Preamble (Evaluating Study Methods and Quality); this assessment did develop study evaluation tables. However, study quality was assessed when determining hazard and identifying which studies were suitable for dose-response analyses. This assessment addresses peer-review comments and retains the structure of the peer-review draft, to maintain fidelity with what the peer reviewers saw. Implementation of systematic review is a process of continuous

improvement subject to periodic review by the Chemical Assessment Advisory Committee (of EPA's Science Advisory Board). This assessment represents a step in the evolution of the IRIS Program.

For additional information about this assessment or for general questions regarding IRIS, please contact EPA's IRIS Hotline at 202-566-1676 (phone), 202-566-1749 (fax), or hotline.iris@epa.gov.

**Assessments by Other National and International Health Agencies**

Toxicity information on 1,2,4-TMB, 1,2,3-TMB, and 1,3,5-TMB has been evaluated by the National Institute for Occupational Safety and Health (NIOSH) and the National Advisory Committee for Acute Exposure Guideline Levels (AEGLs) for Hazardous Substances. The results of these assessments are summarized in Appendix B (Table B-1). It is important to recognize that these assessments may have been prepared for different purposes and may utilize different methods, and that newer studies may be included in the IRIS assessment.

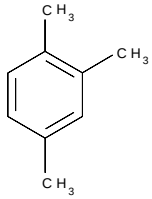
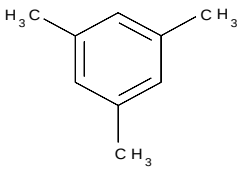
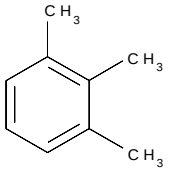
**Chemical Properties and Uses**

TMBs are aromatic hydrocarbons with three methyl groups attached to a benzene ring and the chemical formula $C_9H_{12}$. The chemical and physical properties of the TMB isomers are similar to one another. TMBs are colorless, flammable liquids with a strong aromatic odor; an odor threshold of 0.4 parts per million (ppm) of air has been reported (U.S. EPA, 1994a). They are insoluble in water but miscible with organic solvents such as ethyl alcohol, benzene, and ethyl ether (OSHA, 1996). Production and use of TMBs may result in their release to the environment through various waste streams. If released to the atmosphere, 1,2,3-TMB, 1,2,4-TMB, and 1,3,5-TMB will exist solely in the vapor phase in the atmosphere under ambient conditions, based on measured vapor pressures of 1.69, 2.10, and 2.48 mm Hg at 25°C, respectively (HSDB, 2011a, b, c). All three isomers are expected to have limited mobility through soil based on their log $K_{oc}$ values, but are expected to volatilize from both moist and dry soil surfaces and surface waters based on their respective Henry's law constants and vapor pressures. Degradation of TMB isomers in the atmosphere occurs by reaction with hydroxyl radicals; the half-life is 11–12 hours (HSDB, 2011a, b, c). Non-volatilized TMBs may be subject to biodegradation under aerobic conditions (HSDB, 2011a, b, c). The estimated bioconcentration factors (133–439) and high volatility of TMBs suggest that bioaccumulation of these chemicals will not be significant (U.S. EPA, 1987). Additional information on the chemical identities and physicochemical properties of TMBs is listed in Table P-1.

The commercially available substance known as trimethylbenzene, Chemical Abstracts Service Registry Number (CASRN) 25551-13-7, is a mixture of three isomers in various proportions, namely CASRN 526-73-8 (1,2,3-TMB or hemimellitene), CASRN 95-63-6 (1,2,4-TMB or pseudocumene), and CASRN 108-67-8 (1,3,5-TMB or mesitylene). Production of TMB isomers occurs during petroleum refining, and 1,2,4-TMB individually makes up approximately 40% of the C9 aromatic fraction (i.e., aromatic hydrocarbons with nine carbons) (U.S. EPA, 1994a). The domestic production of the C9 fraction in 1991 was estimated to be approximately 80 billion

pounds (40 million tons) (U.S. EPA, 1994a). Vehicle emissions are a major anthropogenic source of TMBs, due to the widespread use of the C9 fraction as a component of gasoline (U.S. EPA, 1994a). Other uses of TMBs include solvents in research and industry, dyestuff intermediate, paint thinner, and as an ultraviolet oxidation stabilizer for plastics (HSDB, 2011b, c).

**Table P-1. Physical properties and chemical identities of TMB isomers**

| CASRN | 95-63-6 | 108-67-8 | 526-73-8 |
|---|---|---|---|
| Synonym(s) | **1,2,4-Trimethylbenzene, pseudocumene, asymmetrical trimethylbenzene** | **1,3,5-Trimethylbenzene, mesitylene, symmetrical trimethylbenzene** | **1,2,3-Trimethylbenzene, hemimellitene, hemellitol, pseudocumol** |
| Molecular formula | $C_9H_{12}$ | | |
| Molecular weight | 120.19 | | |
| Chemical structure |  |  |  |
| Melting point, °C | −43.8 | −44.8 | −25.4 |
| Boiling point, °C @ 760 mm Hg | 168.9 | 164.7 | 176.1 |
| Vapor pressure, mm Hg @ 25°C | 2.10 | 2.48 | 1.69 |
| Density, g/mL at 20 °C | 0.8758 | 0.8637 | 0.8944 |
| Flashpoint, °C | 44 | 50 | 44 |
| Water solubility, mg/L at 25 °C | 57 | 48.2 | 75.2 |
| Other solubilities | Ethanol, benzene, ethyl ether, acetone, petroleum ether | Alcohol, ether, benzene, acetone, oxygenated and aromatic solvents | Ethanol, acetone, benzene, petroleum ether |
| Henry's law constant, atm $m^3$/mol | $6.16 \times 10^{-3}$ | $8.77 \times 10^{-3}$ | $4.36 \times 10^{-3}$ |
| Log $K_{OW}$ | 3.78 | 3.42 | 3.66 |
| Log $K_{OC}$ | 2.73 | 2.70−3.13 | 2.80−3.04 |
| Bioconcentration factor | 439 | 234 | 133−259 |
| Conversion factors | 1 ppm = 4.92 mg/$m^3$<br>1 mg/$m^3$ = 0.2 ppm | | |

Sources: HSDB (2011a); HSDB (2011b); HSDB (2011c); U.S. EPA (1987).

# PREAMBLE TO IRIS TOXICOLOGICAL REVIEWS

*The Preamble summarizes the objectives and scope of the IRIS program, general principles and systematic review procedures used in developing IRIS assessments, and the overall development process and document structure.*

## 1. Objectives and Scope of the IRIS Program

Soon after EPA was established in 1970, it was at the forefront of developing risk assessment as a science and applying it in support of actions to protect human health and the environment. EPA's IRIS program[1] contributes to this endeavor by reviewing epidemiologic and experimental studies of chemicals in the environment to identify adverse health effects and characterize exposure–response relationships. Health agencies worldwide use IRIS assessments, which are also a scientific resource for researchers and the public.

IRIS assessments cover the hazard identification and dose–response steps of risk assessment. Exposure assessment and risk characterization are outside the scope of IRIS assessments, as are political, economic, and technical aspects of risk management. An IRIS assessment may cover one chemical, a group of structurally or toxicologically related chemicals, or a chemical mixture. Exceptions outside the scope of the IRIS program are radionuclides, chemicals used only as pesticides, and the "criteria air pollutants" (particulate matter, ground-level ozone, carbon monoxide, sulfur oxides, nitrogen oxides, and lead).

Enhancements to the IRIS program are improving its science, transparency, and productivity. To improve the science, the IRIS program is adapting and implementing principles of systematic review (i.e., using explicit methods to identify, evaluate, and synthesize study findings). To increase transparency, the IRIS program discusses key science issues with the scientific community and the public as it begins an assessment. External peer review, independently managed and in public, improves both science and transparency. Increased productivity requires that assessments be concise, focused on EPA's needs, and completed without undue delay.

IRIS assessments follow EPA guidance[2] and standardized practices of systematic review. This Preamble summarizes and does not change IRIS operating procedures or EPA guidance.

Periodically, the IRIS program asks for nomination of agents for future assessment or reassessment. Selection depends on EPA's priorities, relevance to public health, and availability of pertinent studies. The IRIS multiyear agenda[3] lists upcoming assessments. The IRIS program may also assess other agents in anticipation of public health needs.

## 2. Planning an Assessment: Scoping, Problem Formulation, and Protocols

Early attention to planning ensures that IRIS assessments meet their objectives and properly frame science issues.

***Scoping*** refers to the first step of planning, where the IRIS program consults with EPA's program and regional offices to ascertain their needs. Scoping specifies the agents an

[1]IRIS program website: http://www.epa.gov/iris/.
[2]EPA guidance documents: http://www.epa.gov/iris/basic-information-about-integrated-risk-information-system#guidance/.
[3]IRIS multiyear agenda: https://www.epa.gov/iris/iris-agenda.

assessment will address, routes and durations of exposure, susceptible populations and lifestages, and other topics of interest.

***Problem formulation*** refers to the science issues an assessment will address and includes input from the scientific community and the public. A preliminary literature survey, beginning with secondary sources (e.g., assessments by national and international health agencies and comprehensive review articles), identifies potential health outcomes and science issues. It also identifies related chemicals (e.g., toxicologically active metabolites and compounds that metabolize to the chemical of interest).

Each IRIS assessment comprises multiple systematic reviews for multiple health outcomes. It also evaluates hypothesized mechanistic pathways and characterizes exposure–response relationships. An assessment may focus on important health outcomes and analyses rather than expand beyond what is necessary to meet its objectives.

***Protocols*** refer to the systematic review procedures planned for use in an assessment. They include strategies for literature searches, criteria for study inclusion or exclusion, considerations for evaluating study methods and quality, and approaches to extracting data. Protocols may evolve as an assessment progresses and new agent-specific insights and issues emerge.

## 3. Identifying and Selecting Pertinent Studies

IRIS assessments conduct systematic literature searches with criteria for inclusion and exclusion. The objective is to retrieve the pertinent primary studies (i.e., studies with original data on health outcomes or their mechanisms). *PECO statements* (Populations, Exposures, Comparisons, Outcomes) govern the literature searches and screening criteria. "Populations" and animal species generally have no restrictions. "Exposures" refers to the agent and related chemicals identified during scoping and problem formulation and may consider route, duration, or timing of exposure. "Comparisons" means studies that allow comparison of effects across different levels of exposure. "Outcomes" may become more specific (e.g., from "toxicity" to "developmental toxicity" to "hypospadias") as an assessment progresses.

For studies of absorption, distribution, metabolism, and elimination, the first objective is to create an inventory of pertinent studies. Subsequent sorting and analysis facilitates characterization and quantification of these processes.

Studies on mechanistic events can be numerous and diverse. Here, too, the objective is to create an inventory of studies for later sorting to support analyses of related data. The inventory also facilitates generation and evaluation of hypothesized mechanistic pathways.

The IRIS program posts initial protocols for literature searches on its website and adds search results to EPA's HERO database.[4] Then the IRIS program takes extra steps to ensure identification of pertinent studies: by encouraging the scientific community and the public to identify additional studies and ongoing research; by searching for data submitted under the Toxic Substances Control Act or the Federal Insecticide, Fungicide, and Rodenticide Act; and by considering late-breaking studies that would impact the credibility of the conclusions, even during the review process.[5]

## 4. Evaluating Study Methods and Quality

IRIS assessments evaluate study methods and quality, using uniform approaches for each group of similar studies. The objective is that subsequent syntheses can weigh study results on their merits. Key concerns are potential *bias* (factors that affect the magnitude or direction of an effect) and *insensitivity* (factors that limit the ability of a study to detect a true effect).

[4]Health and Environmental Research Online: https://hero.epa.gov/hero/.
[5]IRIS "stopping rules": https://www.epa.gov/sites/production/files/2014-06/documents/iris_stoppingrules.pdf.

For human and animal studies, the evaluation of study methods and quality considers study design, exposure measures, outcome measures, data analysis, selective reporting, and study sensitivity. For human studies, this evaluation also considers selection of participant and referent groups and potential confounding. Emphasis is on discerning bias that could substantively change an effect estimate, considering also the expected direction of the bias. Low sensitivity is a bias towards the null.

Study-evaluation considerations are specific to each study design, health effect, and agent. Subject-matter experts evaluate each group of studies to identify characteristics that bear on the informativeness of the results. For carcinogenicity, neurotoxicity, reproductive toxicity, and developmental toxicity, there is EPA guidance for study evaluation (U.S. EPA, 2005a, 1998, 1996, 1991). As subject-matter experts examine a group of studies, additional agent-specific knowledge or methodologic concerns may emerge and a second pass become necessary.

Assessments use evidence tables to summarize the design and results of pertinent studies. If tables become too numerous or unwieldy, they may focus on effects that are more important or studies that are more informative.

The IRIS program posts initial protocols for study evaluation on its website, then considers public input as it completes this step.

## 5. Integrating the Evidence of Causation for Each Health Outcome

**Synthesis within lines of evidence.** For each health outcome, IRIS assessments synthesize the human evidence and the animal evidence, augmenting each with informative subsets of mechanistic data. Each synthesis considers aspects of an association that may suggest causation: consistency, exposure–response relationship, strength of association, temporal relationship, biological plausibility, coherence, and "natural experiments" in humans (U.S. EPA, 1994, §2.1.3) (U.S. EPA, 2005a, §2.5).

Each synthesis seeks to reconcile ostensible inconsistencies between studies, taking into account differences in study methods and quality. This leads to a distinction between *conflicting evidence* (unexplained positive and negative results in similarly exposed human populations or in similar animal models) and *differing results* (mixed results attributable to differences between human populations, animal models, or exposure conditions) (U.S. EPA, 2005a, §2.5).

Each synthesis of human evidence explores alternative explanations (e.g., chance, bias, or confounding) and determines whether they may satisfactorily explain the results. Each synthesis of animal evidence explores the potential for analogous results in humans. Coherent results across multiple species increase confidence that the animal results are relevant to humans.

Mechanistic data are useful to augment the human or animal evidence with information on precursor events, to evaluate the human relevance of animal results, or to identify susceptible populations and lifestages. An agent may operate through multiple mechanistic pathways, even if one hypothesis dominates the literature (U.S. EPA, 2005a, §2.4.3.3).

**Integration across lines of evidence.** For each health outcome, IRIS assessments integrate the human, animal, and mechanistic evidence to answer the question: *What is the nature of the association between exposure to the agent and the health outcome?*

For cancer, EPA includes a standardized hazard descriptor in characterizing the strength of the evidence of causation. The objective is to promote clarity and consistency of conclusions across assessments (U.S. EPA, 2005a, §2.5).

*Carcinogenic to humans:* convincing epidemiologic evidence of a causal association; or strong human evidence of cancer or its key precursors, extensive animal evidence, identification of mode-of-action and its key precursors in animals, and strong evidence that they are anticipated in humans.

*Likely to be carcinogenic to humans:* evidence that demonstrates a potential hazard to humans. Examples include a plausible association in humans with supporting experimental evidence, multiple positive

results in animals, a rare animal response, or a positive study strengthened by other lines of evidence.

*Suggestive evidence of carcinogenic potential:* evidence that raises a concern for humans. Examples include a positive result in the only study, or a single positive result in an extensive database.

*Inadequate information to assess carcinogenic potential:* no other descriptors apply. Examples include little or no pertinent information, *conflicting evidence*, or negative results not sufficiently robust for *not likely*.

*Not likely to be carcinogenic to humans:* robust evidence to conclude that there is no basis for concern. Examples include no effects in well-conducted studies in both sexes of multiple animal species, extensive evidence showing that effects in animals arise through modes-of-action that do not operate in humans, or convincing evidence that effects are not likely by a particular exposure route or below a defined dose.

If there is credible evidence of carcinogenicity, there is an evaluation of mutagenicity, because this influences the approach to dose–response assessment and subsequent application of adjustment factors for exposures early in life (U.S. EPA, 2005a, §3.3.1, §3.5), (U.S. EPA, 2005b, §5).

## 6. Selecting Studies for Derivation of Toxicity Values

The purpose of toxicity values (slope factors, unit risks, reference doses, reference concentrations; see section 7) is to estimate exposure levels likely to be without appreciable risk of adverse health effects. EPA uses these values to support its actions to protect human health.

The health outcomes considered for derivation of toxicity values may depend on the hazard descriptors. For example, IRIS assessments generally derive cancer values for agents that are *carcinogenic* or *likely to be carcinogenic*, and sometimes for agents with *suggestive evidence* (U.S. EPA, 2005a, §3).

Derivation of toxicity values begins with a new evaluation of studies, as some studies used qualitatively for hazard identification may not be useful quantitatively for exposure–response assessment. Quantitative analyses require quantitative measures of exposure and response. An assessment weighs the merits of the human and animal studies, of various animal models, and of different routes and durations of exposure (U.S. EPA, 1994, §2.1). Study selection is not reducible to a formula, and each assessment explains its approach.

Other biological determinants of study quality include appropriate measures of exposure and response, investigation of early effects that precede overt toxicity, and appropriate reporting of related effects (e.g., combining effects that comprise a syndrome, or benign and malignant tumors in a specific tissue).

Statistical determinants of study quality include multiple levels of exposure (to characterize the shape of the exposure–response curve) and adequate exposure range and sample sizes (to minimize extrapolation and maximize precision) (U.S. EPA, 2012, §2.1).

Studies of low sensitivity may be less useful if they fail to detect a true effect or yield toxicity values with wide confidence limits.

## 7. Deriving Toxicity Values

**General approach.** EPA guidance describes a two-step approach to dose–response assessment: analysis in the range of observation, then extrapolation to lower levels. Each toxicity value pertains to a route (e.g., oral, inhalation, dermal) and duration or timing of exposure (e.g., chronic, subchronic, gestational) (U.S. EPA, 2002, §4).

IRIS assessments derive a candidate value from each suitable data set. Consideration of candidate values yields a toxicity value for each organ or system. Consideration of the organ/system-specific values results in the selection of an overall toxicity value to cover all health outcomes. The organ/system-specific values are useful for subsequent cumulative risk assessments that consider the combined effect of

multiple agents acting at a common anatomical site.

**Analysis in the range of observation.** Within the observed range, the preferred approach is modeling to incorporate a wide range of data. Toxicokinetic modeling has become increasingly common for its ability to support target-dose estimation, cross-species adjustment, or exposure-route conversion. If data are too limited to support toxicokinetic modeling, there are standardized approaches to estimate daily exposures and scale them from animals to humans (U.S. EPA, 1994, §3), (U.S. EPA, 2005a, §3.1), (U.S. EPA, 2011, 2006).

For human studies, an assessment may develop exposure–response models that reflect the structure of the available data (U.S. EPA, 2005a, §3.2.1). For animal studies, EPA has developed a set of empirical ("curve-fitting") models[6] that can fit typical data sets (U.S. EPA, 2005a, §3.2.2). Such modeling yields a *point of departure*, defined as a dose near the lower end of the observed range, without significant extrapolation to lower levels (e.g., the estimated dose associated with an extra risk of 10% for animal data or 1% for human data, or their 95% lower confidence limits)(U.S. EPA, 2005a, §3.2.4), (U.S. EPA, 2012, §2.2.1).

When justified by the scope of the assessment, toxicodynamic ("biologically based") modeling is possible if data are sufficient to ascertain the key events of a mode-of-action and to estimate their parameters. Analysis of model uncertainty can determine the range of lower doses where data support further use of the model (U.S. EPA, 2005a, §3.2.2, §3.3.2).

For a group of agents that act at a common site or through common mechanisms, an assessment may derive relative potency factors based on relative toxicity, rates of absorption or metabolism, quantitative structure–activity relationships, or receptor-binding characteristics (U.S. EPA, 2005a, §3.2.6).

**Extrapolation: slope factors and unit risks.** An *oral slope factor* or an *inhalation unit risk* facilitates subsequent estimation of human cancer risks. Extrapolation proceeds linearly (i.e., risk proportional to dose) from the point of departure to the levels of interest. This is appropriate for agents with direct mutagenic activity. It is also the default if there is no established mode-of-action (U.S. EPA, 2005a, §3.3.1, §3.3.3).

Differences in susceptibility may warrant derivation of multiple slope factors or unit risks. For early-life exposure to carcinogens with a mutagenic mode-of-action, EPA has developed default *age-dependent adjustment factors* for agents without chemical-specific susceptibility data (U.S. EPA, 2005a, §3.5), (U.S. EPA, 2005b, §5).

If data are sufficient to ascertain the mode-of-action and to conclude that it is not linear at low levels, extrapolation may use the reference-value approach (U.S. EPA, 2005a, §3.3.4).

**Extrapolation: reference values.** An *oral reference dose* or an *inhalation reference concentration* is an estimate of human exposure (including in susceptible populations) likely to be without appreciable risk of adverse health effects over a lifetime (U.S. EPA, 2002, §4.2). Reference values generally cover effects other than cancer. They are also appropriate for carcinogens with a nonlinear mode-of-action.

Calculation of reference values involves dividing the point of departure by a set of *uncertainty factors* (each typically 1, 3, or 10, unless there are adequate chemical-specific data) to account for different sources of uncertainty and variability (U.S. EPA, 2002, §4.4.5), (U.S. EPA, 2014).

*Human variation:* An uncertainty factor covers susceptible populations and lifestages that may respond at lower levels, unless the data originate from a susceptible study population.

*Animal-to-human extrapolation:* For reference values based on animal results, an uncertainty factor reflects cross-species differences, which may cause humans to respond at lower levels.

*Subchronic-to-chronic exposure:* For chronic reference values based on subchronic studies, an uncertainty factor reflects the likelihood that a lower level over a longer duration may induce a similar response. This

[6]Benchmark Dose Software: http://www.epa.gov/bmds/.

factor may not be necessary for reference values of shorter duration.

*Adverse-effect level to no-observed-adverse-effect level:* For reference values based on a lowest-observed-adverse-effect level, an uncertainty factor reflects a level judged to have no observable adverse effects.

*Database deficiencies:* If there is concern that future studies may identify a more sensitive effect, target organ, population, or lifestage, a *database uncertainty factor* reflects the nature of the database deficiency.

## 8. Process for Developing and Peer-Reviewing IRIS Assessments

The IRIS process (revised in 2009 and enhanced in 2013) involves extensive public engagement and multiple levels of scientific review and comment. IRIS program scientists consider all comments. Materials released, comments received from outside EPA, and disposition of major comments (steps 3, 4, and 6 below) become part of the public record.

**Step 1: Draft development.** As outlined in section 2 of this Preamble, IRIS program scientists specify the scope of an assessment and formulate science issues for discussion with the scientific community and the public. Next, they release initial protocols for the systematic review procedures planned for use in the assessment. IRIS program scientists then develop a first draft, using structured approaches to identify pertinent studies, evaluate study methods and quality, integrate the evidence of causation for each health outcome, select studies for derivation of toxicity values, and derive toxicity values, as outlined in Preamble sections 3–7.

**Step 2: Agency review.** Health scientists across EPA review the draft assessment.

**Step 3: Interagency science consultation.** Other federal agencies and the Executive Office of the President review the draft assessment.

**Step 4: Public comment, followed by external peer review.** The public reviews the draft assessment. IRIS program scientists release a revised draft for independent external peer review. The peer reviewers consider whether the draft assessment assembled and evaluated the evidence according to EPA guidance and whether the evidence justifies the conclusions.

**Step 5: Revise assessment.** IRIS program scientists revise the assessment to address the comments from the peer review.

**Step 6: Final agency review and interagency science discussion.** The IRIS program discusses the revised assessment with EPA's program and regional offices and with other federal agencies and the Executive Office of the President.

**Step 7: Post final assessment.** The IRIS program posts the completed assessment and a summary on its website.

## 9. General Structure of IRIS Assessments

**Main text.** IRIS assessments generally comprise two major sections: (1) Hazard Identification and (2) Dose–Response Assessment. Section 1.1 briefly reviews chemical properties and toxicokinetics to describe the disposition of the agent in the body. This section identifies related chemicals and summarizes their health outcomes, citing authoritative reviews. If an assessment covers a chemical mixture, this section discusses environmental processes that alter the mixtures humans encounter and compares them to mixtures studied experimentally.

Section 1.2 includes a subsection for each major health outcome. Each subsection discusses the respective literature searches and study considerations, as outlined in Preamble sections 3 and 4, unless covered in the front matter. Each subsection concludes with evidence synthesis and integration, as outlined in Preamble section 5.

Section 1.3 links health hazard information to dose–response analyses for each health outcome. One subsection identifies susceptible populations and lifestages, as observed in human

or animal studies or inferred from mechanistic data. These may warrant further analysis to quantify differences in susceptibility. Another subsection identifies biological considerations for selecting health outcomes, studies, or data sets, as outlined in Preamble section 6.

Section 2 includes a subsection for each toxicity value. Each subsection discusses study selection, methods of analysis, and derivation of a toxicity value, as outlined in Preamble sections 6 and 7.

**Front matter.** The Executive Summary provides information historically included in IRIS summaries on the IRIS program website. Its structure reflects the needs and expectations of EPA's program and regional offices.

A section on systematic review methods summarizes key elements of the protocols, including methods to identify and evaluate pertinent studies. The final protocols appear as an appendix.

The Preface specifies the scope of an assessment and its relation to prior assessments. It discusses issues that arose during assessment development and emerging areas of concern.

This Preamble summarizes general procedures for assessments begun after the date below. The Preface identifies assessment-specific approaches that differ from these general procedures.

August 2016

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# EXECUTIVE SUMMARY

***Occurrence and Health Effects***

Trimethylbenzenes (TMBs) are a commercially available mixture of three individual isomers: 1,2,3-TMB, 1,2,4-TMB, and 1,3,5-TMB. TMB isomers are produced during petroleum refining and production of aromatic hydrocarbons with nine carbons (i.e., C9 aromatic fraction). As the vast majority of the C9 fraction is used as a component of gasoline, vehicle emissions are expected to be the major anthropogenic source of TMBs. TMBs are volatile hydrocarbons, and humans are thus exposed to these isomers primarily through breathing air containing TMB vapors, although ingestion through food or drinking water is also possible.

Effects on the nervous, respiratory, and hematological (i.e., blood) systems have been reported in occupationally- and residentially-exposed humans, but these effects were observed following exposure to complex mixtures containing TMB isomers, thus making it difficult to determine the contribution of each TMB isomer to the observed health effects. Health effects that are roughly analogous to those seen in humans have been observed in animals exposed to the individual isomers. Effects on the nervous system, including cognitive effects and decreased pain sensitivity, are the most widely observed effects in animals. Effects on other systems, including the respiratory and hematological systems, have also been observed in animals. Both 1,2,4-TMB and 1,3,5-TMB have been observed to elicit effects on pregnant animals and developing fetuses, but at exposure levels greater than those that cause effects on the nervous system. There is inadequate information to evaluate the carcinogenicity of TMBs.

**Effects Other Than Cancer Following Inhalation Exposure**

The relationship between exposure to 1,2,3-TMB, 1,2,4-TMB, and 1,3,5-TMB and health effects has been evaluated in studies of (1) exposed human adults, (2) animals exposed via inhalation for acute, short-term, and subchronic durations, and (3) animals exposed gestationally via inhalation.

Human studies included occupational exposure to various solvent mixtures containing TMBs. Health effects noted in these studies were eye irritation, neurological effects (hand tremble, abnormal fatigue, lack of coordination), and hematological effects. Residential exposure to mixtures containing 1,2,4-TMB were observed to be associated with asthma. However, as these studies involved exposures to mixtures containing multiple TMB isomers and other volatile organic

compounds (VOCs), it is difficult to ascertain the specific contribution of each TMB isomer to the specific health effects reported. Studies involving controlled exposures of healthy adult volunteers to individual isomers also exist, although these studies generally reported little or no sensory irritation or effects on the respiratory system. One controlled human exposure study reported some deficits in attention following exposure to white spirit, a complex mixture containing 1,2,4-TMB.

Animal inhalation studies included acute and short-term studies of TMBs that reported respiratory irritation (decreased respiration rates) and neurological effects (decreased pain sensitivity, altered cognitive function, and decreased anxiety and/or increased motor function) that are consistent with effects seen in human studies. Four subchronic inhalation studies for 1,2,3-TMB and 1,2,4-TMB observed exposure-response effects in multiple systems, including the nervous, hematological, and respiratory systems. In these studies, disturbances in central nervous system (CNS) function, including decreased pain sensitivity and decreased neuromuscular function and coordination, appear to be the most sensitive endpoints following exposure to 1,2,3-TMB or 1,2,4-TMB. No subchronic studies were found that investigated exposure to 1,3,5-TMB. One developmental toxicity study observed maternal and fetal toxicity (i.e., decreased maternal weight gain and fetal weight) following exposure to either 1,2,4-TMB or 1,3,5-TMB; other indices of fetal toxicity (i.e., fetal death and malformations) were not affected by exposure.

**Inhalation Reference Concentration (RfC) for TMBs for Effects Other Than Cancer**

The RfC for TMBs was derived using benchmark dose (BMD) modeling coupled with physiologically-based pharmacokinetic (PBPK) modeling or default dosimetric methods. BMD modeling was conducted using external exposure concentrations as the dose inputs and either a benchmark response (BMR) level of 5% change (fetal weight) or 1 standard deviation (SD) of the control mean (all other endpoints). Once a lower confidence limit on the benchmark dose (BMDL) (or a no-observed-adverse-effect level [NOAEL] or lowest-observed-adverse-effect level [LOAEL] in cases where no models fit the data) was identified as the point of departure (POD), a human equivalent concentration (HEC) was calculated for each endpoint using either a PBPK model (1,2,4-TMB) or default dosimetric adjustments (1,2,3-TMB and 1,3,5-TMB).

To each HEC, a composite uncertainty factor (UF) was applied to account for uncertainties in the TMB database: 3 to account for uncertainty in extrapolating from laboratory animals to humans (interspecies variability), 10 to account for variation in susceptibility among members of the human population (interindividual variability), 3 to account for subchronic-to-chronic extrapolation due to the use of a subchronic study, and 3 to account for deficiencies in the database (no TMB-specific developmental neurotoxicity studies were available). Full details of the selection and application of the UFs are available in Section 2.1.3. Dividing the candidate HECs by this composite UF of 300 yielded the organ/system-specific RfCs presented in Table ES-1.

**Table ES-1. Organ/system-specific chronic RfCs for individual TMB isomers**

| Effect | Isomer | Basis | RfC ($mg/m^3$) | Composite UF | Exposure description | Confidence |
|---|---|---|---|---|---|---|
| Neurological | 1,2,4-TMB | Decreased pain sensitivity | $6 \times 10^{-2}$ | 300 | Subchronic | Low to medium |
| | 1,2,3-TMB | | $5 \times 10^{-2}$ | 300 | Subchronic | Low to medium |
| Hematological | 1,2,4-TMB | Decreased clotting time | $8 \times 10^{-2}$ | 300 | Subchronic | Low to medium |
| | 1,2,3-TMB | Decreased segmented neutrophils | $6 \times 10^{-2}$ | 300 | Subchronic | Low to medium |
| Respiratory | 1,2,4-TMB | Inflammatory lung lesions | $2 \times 10^{-1}$ | 300 | Subchronic | Low to medium |
| | 1,2,3-TMB | | $2 \times 10^{-1}$ | 300 | Subchronic | Low to medium |
| Developmental | 1,2,4-TMB | Decreased fetal weight | 4 | 100 | Gestational | Low to medium |
| | 1,3,5-TMB | | 4 | 100 | Gestational | Low to medium |
| Maternal | 1,2,4-TMB | Decreased maternal weight | 3 | 300 | Subchronic | Low to medium |
| | 1,3,5-TMB | | $4 \times 10^{-1}$ | 300 | Subchronic | Low to medium |
| **Chronic Overall RfC (Neurological)** | **All TMB isomers** | **Decreased pain sensitivity** | **$6 \times 10^{-2}$** | **300** | **Subchronic** | **Low to medium** |

Neurotoxicity is the most consistently observed endpoint in the toxicological database for TMBs, and decreased pain sensitivity was observed in multiple studies following exposures to 1,2,3- or 1,2,4-TMB for short-term or subchronic durations. Given the consistency of this effect and the determination that decreased pain sensitivity is an appropriate adverse effect with which to derive reference values (see Section 2.1.5), in accordance with the EPA's *Guidelines for Neurotoxicity Risk Assessment* (U.S. EPA, 1998), decreased pain sensitivity was selected as the critical effect and Korsak and Rydzyński (1996) was selected as the principal study for derivation of the RfC for TMBs. No subchronic study was available that investigated neurotoxicity endpoints following exposure to 1,3,5-TMB, resulting in the lack of an isomer-specific neurotoxicity RfC for this isomer. However, as discussed in Section 1.2.7, the available toxicological database for all three isomers, across all exposure durations, indicates there are important similarities in the isomers' neurotoxicity that are supportive of an RfC for 1,3,5-TMB that is not substantially different than the RfC derived for other TMB isomers. Also supporting this conclusion is the observation that TMB isomers display important similarities with regard to chemical properties and toxicokinetics, including similarities in blood:air partition coefficients, respiratory uptake, and absorption into the bloodstream (see Section 1.2.7 and Appendices C.1 and C.2). These similarities support the conclusion that an RfC for 1,3,5-TMB would be similar to those calculated for 1,2,3- or 1,2,4-TMB. The RfC for 1,2,4-TMB was selected over the RfC for 1,2,3-TMB as the RfC for the entire TMB database due to increased

confidence in that it was calculated via the application of a validated PBPK model, whereas the 1,2,3-TMB value was estimated using default dosimetric methods. **Therefore, the chronic RfC for all TMBs was set at $6 \times 10^{-2}$ mg/m$^3$ based on neurological effects following exposure to 1,2,4-TMB.** However, this overall RfC for TMBs can be used for any TMB isomer alone, or in situations when individuals are exposed to a mixture of TMB isomers. The individual organ- or system-specific RfCs may be useful for subsequent cumulative risk assessments that consider the combined effect of multiple agents acting at a common site.

In addition to providing an RfC for chronic exposures in multiple systems, this document also provides an RfC for subchronic-duration exposures. In the case of TMBs, all of the studies used to calculate the chronic RfCs were subchronic or gestational in duration. Therefore, the methods to calculate subchronic RfCs are identical to those used for calculation of chronic RfCs, minus the application of a subchronic-to-chronic UF ($UF_S$) (see Table ES-1). It should be noted that the subchronic RfC values for the developing fetus are identical to the chronic RfC values for the developing fetus as gestation represents a critical window of susceptibility and no $UF_S$ was applied to account for less-than-chronic exposure in either case. The subchronic inhalation RfC is intended for use with exposures for more than 30 days, up to approximately 10% of the lifespan in humans. **The subchronic RfC for TMBs was set to $2 \times 10^{-1}$ mg/m$^3$ based on neurological effects following exposure to 1,2,4-TMB.**

**Table ES-2. Organ/system-specific subchronic RfCs for individual TMB isomers**

| Effect | Isomer | Basis | RfC (mg/m$^3$) | Composite UF | Exposure description | Confidence |
|---|---|---|---|---|---|---|
| Neurological | 1,2,4-TMB | Decreased pain sensitivity | $2 \times 10^{-1}$ | 100 | Subchronic | Low to medium |
| | 1,2,3-TMB | | $2 \times 10^{-1}$ | 100 | Subchronic | Low to medium |
| Hematological | 1,2,4-TMB | Decreased clotting time | $2 \times 10^{-1}$ | 100 | Subchronic | Low to medium |
| | 1,2,3-TMB | Decreased segmented neutrophils | $2 \times 10^{-1}$ | 100 | Subchronic | Low to medium |
| Respiratory | 1,2,4-TMB | Inflammatory lung lesions | $6 \times 10^{-1}$ | 100 | Subchronic | Low to medium |
| | 1,2,3-TMB | | $6 \times 10^{-1}$ | 100 | Subchronic | Low to medium |
| Developmental | 1,2,4-TMB | Fetal weight | 4 | 100 | Gestational | Low to medium |
| | 1,3,5-TMB | | 4 | 100 | Gestational | Low to medium |
| Maternal | 1,2,4-TMB | Decreased maternal weight | 8 | 100 | Subchronic | Low to medium |
| | 1,3,5-TMB | | 1 | 100 | Subchronic | Low to medium |
| **Subchronic Overall RfC (Neurological)** | **All TMB isomers** | **Decreased pain sensitivity** | **$2 \times 10^{-1}$** | **100** | **Subchronic** | **Low to medium** |

**Confidence in the Chronic Inhalation RfC for 1,2,4-TMB**

A confidence level of high, medium, or low is assigned to the study used to derive the RfC, the overall database, and the RfC itself, as described in Section 4.3.9.2 of EPA's *Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry (U.S. EPA, 1994b)*.

Confidence in the study from which the critical effect was identified is low to medium. The study is a peer-reviewed study that utilized three dose groups plus untreated controls, employed an appropriate number of animals per dose group, and performed appropriate statistical analyses. However, sources of uncertainty exist that reduce confidence in this study.

One area of uncertainty regarding this study is the lack of reported actual concentrations. However, as the methods by which the test atmosphere was generated and analyzed were reported in sufficient detail, and given the fact that this laboratory has used this methodology in subsequent studies and achieved appropriate actual concentrations (i.e., within 10% of target concentrations), the concern regarding the lack of reported actual concentrations is reduced. Another source of uncertainty is the fact that the principal study does not explicitly state that the reported measures of variance in Table 1 of that reference are SDs. However, careful analysis of the reported levels of variance and magnitude of statistical significance indicate that the measures of variance are SDs. Supporting this conclusion is the observation that all other papers from this laboratory report variance as SDs. The critical effect on which the RfC is based is well-supported as the weight of evidence for TMB-induced neurotoxicity is coherent across species (i.e., human, mouse, and rat), coherent across isomers, and consistent across multiple exposure durations (i.e., acute, short-term, and subchronic).

The database for TMBs includes acute, short-term, subchronic, and developmental toxicity studies in rats and mice. However, confidence in the overall database is low to medium because it lacks chronic and developmental neurotoxicity studies, and the studies supporting the critical effect predominantly come from the same research institute. The overall confidence in the RfC for TMBs is low to medium.

**Effects Other Than Cancer Observed Following Oral Exposure**

Only one subchronic study was identified that examined the effects of oral exposure to 1,3,5-TMB. Effects in the hematological system, including changes in clinical chemistry parameters and differential white blood cell (WBC) numbers, were observed following exposure to 1,3,5-TMB via gavage in rats. Altered organ weights were also observed in multiple systems (kidneys, liver). The alterations to clinical chemistry parameters and organ weights were observed in the absence of histopathological changes in relevant systems, and were thus considered to be compensatory in nature. Discounting effects that could be non-adverse or compensatory in nature left an observed increase in monocytes in male rats as the only statistically significant effect on which to base the reference dose (RfD) derivation. While a slight increase in monocytes may be of questionable

adversity if taken with no context of the larger TMB database, a number of endpoints involving the alteration of WBC counts have been observed in the inhalation toxicity database. It was therefore deemed that the observed increase in monocytes following oral exposures was possibly indicative of an underlying toxicity to the hematological system also evident following inhalation exposure.

**Oral Reference Dose (RfD) for TMBs for Effects Other Than Cancer**

The RfD for TMBs was derived using BMD modeling coupled with default dosimetric methods. BMD modeling was conducted using external exposure concentrations as the dose inputs and a BMR level of 1 SD of the control mean. Once a BMDL was identified as the POD, a human equivalent dose (HED) of 3.0 mg/kg-day was calculated for increased monocytes using default dosimetric adjustments (i.e., body weight to the ¾ power).

To the estimated HED, a composite UF was applied to account for uncertainties in the TMB database: 3 to account for uncertainty in extrapolating from laboratory animals to humans (interspecies variability), 10 to account for variation in susceptibility among members of the human population (interindividual variability), 3 to account for subchronic-to-chronic extrapolation due to the use of a subchronic study, and 3 to account for deficiencies in the database (no TMB-specific developmental neurotoxicity studies were available). Full details of the selection and application of the UFs are available in Section 2.2.3. **Dividing the HED by this composite UF of 300 yielded an RfD of $1 \times 10^{-2}$ mg/kg-day that can be applied to any TMB isomer individually or to mixtures of TMB isomers.**

In addition to the RfD calculated for TMBs from oral data, an RfD was calculated from inhalation data using a route-to-route extrapolation to address the lack of suitable neurotoxicity data in the oral TMB database. It is clear from the inhalation database for TMB that neurotoxicity is an important endpoint for derivation of reference values, especially given the consistency with which neurotoxicity is observed in the TMB database, across all isomers following acute oral and acute, short-term, and subchronic inhalation exposures. Ultimately, the fact that oral and inhalation neurotoxic endpoints are comparable, and that neurotoxic endpoints resulted in the most strongly supported RfCs in the inhalation database, it is reasonable to expect that neurotoxicity-based PODs would be critical for deriving RfDs. The available database for 1,2,4-TMB supports the use of route-to-route extrapolation; sufficient evidence exists that demonstrates similar qualitative profiles of metabolism (i.e., observation of dimethylbenzoic and hippuric acid metabolites) and patterns of parent compound distribution across exposure routes (Section C.2, Appendix C).

Therefore, assuming that oral exposure would result in the same systemic effect as inhalation exposure (i.e., altered CNS function, measured as decreased pain sensitivity), an oral exposure component was added to the PBPK model by EPA (Section C.3.3.5, Appendix C), assuming 100% absorption of the ingested 1,2,4-TMB by constant infusion of the oral dose into the liver and an idealized pattern of six ingestion events (see Section 2.2.3). **Using the modified PBPK model resulted in an HED of 3.5 mg/kg-day, which was divided by the composite UF of 300 to estimate an RfD of $1 \times 10^{-2}$ mg/kg-day.** Although identical to the RfD calculated from the oral

1,3,5-TMB data for increased monocytes, this value of $1 \times 10^{-2}$ mg/kg-day was ultimately selected as the RfD for TMB isomers based on multiple lines of evidence in the oral and inhalation database, including commonalities in the pattern of neurotoxic effects observed following oral and inhalation exposures, similarities in blood:air and tissue:air partition coefficients and absorption into the bloodstream between TMB isomers, and qualitative metabolic profiles that suggest that first-pass metabolism through the liver is not expected to differ greatly between the three isomers.

In addition to providing RfDs for effects in the hematological and nervous systems, this document also provides values for subchronic RfD values for exposures that may be of concern in a less-than-lifetime context. In the case of TMBs, the oral 1,3,5-TMB study and the inhalation 1,2,4-TMB study used for the route-to-route extrapolation for the calculation of the chronic RfDs were both of subchronic duration. Therefore, the methods used to calculate subchronic RfDs is identical to that used for calculation of chronic RfCs, minus the application of a $UF_S$. This results in a composite UF of 100 (interspecies UF [$UF_A$] of 3, intraspecies UF [$UF_H$] of 10, $UF_S$ of 1, and database UF [$UF_D$ ] of 3). Dividing the POD for hematological effects (3.01 mg/kg-day) and neurotoxicity effects (3.5 mg/kg-day) by the composite UF of 100 results in RfDs of $3 \times 10^{-2}$ and $4 \times 10^{-2}$ mg/kg-day for decreased monocytes and decreased pain sensitivity, respectively. **The subchronic RfD was set to $4 \times 10^{-2}$ mg/kg-day based on neurological effects following exposure to 1,2,4-TMB**. The subchronic oral RfD is intended for use with exposures for more than 30 days, up to approximately 10% of the lifespan in humans.

**Confidence in the Chronic Oral RfD for 1,2,4-TMB**

The confidence in the oral database for TMB is low because it only contains acute oral studies investigating neurotoxicity endpoints for multiple isomers, and one subchronic study investigating general toxicity endpoints for one isomer (1,3,5-TMB). This database was used to derive an RfD, but given the concern over the lack of a suitable neurotoxicity study, the confidence in this RfD is low. A PBPK model was utilized to perform a route-to-route extrapolation to determine a POD for the derivation of the RfD from inhalation data. The confidence in the study from which the critical effect was identified is low to medium (see Section 2.1.7). The inhalation database for 1,2,4-TMB includes acute, short-term, subchronic, and developmental toxicity studies in rats and mice. However, confidence in the overall database for TMB is low to medium because it lacks chronic and developmental neurotoxicity studies, and the studies supporting the critical effect predominantly come from the same research institute. Reflecting the confidence in the study and the database and the uncertainty surrounding the application of the available PBPK model for the purposes of a route-to-route extrapolation, the overall confidence in the RfD for TMB is low.

**Evidence of Carcinogenicity**

Under EPA's *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005), there is "inadequate information to assess carcinogenic potential" of TMBs. No chronic inhalation studies that investigated cancer outcomes were identified in the literature for 1,2,3-TMB, 1,2,4-TMB, or

1,3,5-TMB. One cancer study in which rats were exposed to 1,2,4-TMB via gavage at one experimental dose of 800 mg/kg-day reported marginal increases in total malignant tumors and head tumors (e.g., neuroesthesioepitheliomas), but provided no statistical analyses of the results. A number of methodological issues limit the utility of this study (e.g., only one dose group and no discussion of histopathological analyses). Therefore, **a quantitative cancer assessment for TMBs was not conducted.**

**Susceptible Populations and Lifestages**

No TMB-specific data that would allow for the identification of populations or lifestages with increased susceptibility to TMB exposure exist. However, some data gleaned from the related compound, toluene, do provide some suggestive evidence that periods in early life represent periods of susceptibility to solvent exposure. Therefore, it can be reasonably assumed that exposures in early life to individual TMB isomers are of particular concern.

# LITERATURE SEARCH STRATEGY | STUDY SELECTION AND EVALUATION

A number of literature searches were conducted to identify references for inclusion in the trimethylbenzene (TMB) assessment. The initial literature search strategy used to identify primary, peer-reviewed literature pertaining to TMBs was conducted using the databases and keywords listed in Table LS-1. References from health assessments developed by other national and international health agencies were also examined. Other peer-reviewed information, including review articles, literature necessary for the interpretation of TMB-induced health effects, and independent analyses of the health effects data were retrieved and included in the assessment where appropriate; these references are included in the primary literature search numbers. The U.S. Environmental Protection Agency (EPA) requested public submissions of additional information in April 2008; no submissions in response to the data call-in were received. The initial literature search was last conducted in December 2011.

**Table LS-1. Details of the initial search strategy employed for TMBs**

| Databases | Terms[a,b] |
|---|---|
| EBSCO DISCOVERY SERVICE:<br>HERO<br>SCI<br>NLM<br>TOXLINE<br>WOS | **Chemical name, CASRN, and synonym search:** 1,2,4-trimethylbenzene OR pseudocumene OR 95-63-6; 1,2,3-trimethylbenzene OR hemimellitene OR 526-73-8; 1,3,5-trimethylbenzene OR mesitylene OR 108-67-8<br>**Keyword search:** neurotoxicity, genotoxicity, developmental toxicity, inflammation, irritation, toxicokinetics, pbpk, mode of action, white spirit, C9, C9 fraction, JP-8<br>**Additional search on specific metabolites:**<br>2,3-dimethylbenzoic acid OR 26998-80-1; 2,3-dimethylhippuric acid OR 187980-99-0;<br>2,4-dimethylbenzoic acid OR 611-01-8; 2,4-dimethylhippuric acid OR 41859-41-0;<br>2,5-dimethylbenzoic acid OR 610-72-0; 2,5-dimethylhippuric acid OR 41859-40-9;<br>2,6-dimethylbenzoic acid OR 632-46-2; 2,6-dimethylhippuric acid OR 187980-98-9;<br>3,4-dimethylbenzoic acid OR 619-04-5; 3,4-dimethylhippuric acid OR 23082-12-4;<br>2,4,5-trimethylphenol OR 496-78-6; 2,3,5-trimethylphenol OR 697-82-5; 2,3,6-trimethylphenol OR 2416-94-6; 2,4,6-trimethylphenol OR 527-60-6; 3,5-dimethylbenzoic acid OR 499-06-9;<br>3,5-dimethylhippuric acid OR 23082-14-6 |

[a]Potentially relevant publications on TMBs were identified through a literature search conducted with the EBSCO Discovery Service feature of Health and Environmental Research Online (HERO), a meta-search engine with access to numerous databases including the Science Citation Index (SCI), Toxicology Literature Online (TOXLINE), National Library of Medicine (NLM, PubMed/Medline), and Web of Science (WOS).

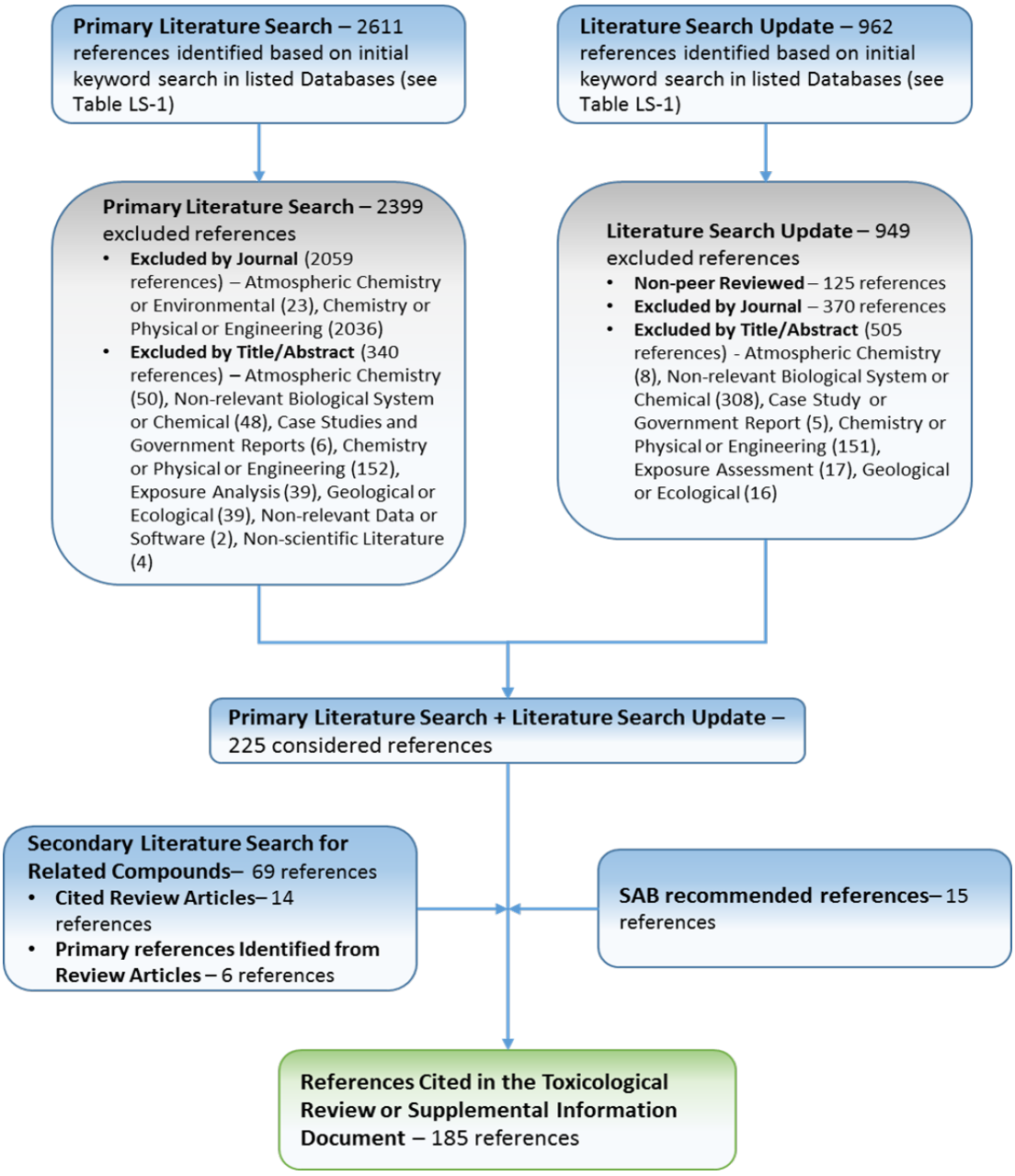
[b]Literature search was performed using related words (i.e., lemmatization) of included search terms. Search terms were entered into the EBSCO Discovery Service portal with no qualifiers, and the results from individual search engines were returned and exported to HERO.

CASRN = Chemical Abstracts Service Registry Number.

Figure LS-1 depicts the literature search and study selection strategy and the number of references obtained at each stage of the literature screening. Selection of studies for inclusion in the Toxicological Review was based on consideration of the extent to which the study was informative and relevant to the assessment and general study quality considerations. In general, the relevance of health effect studies was evaluated as outlined in the Preamble and EPA guidance (*A Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002) and *Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry* (U.S. EPA, 1994b)). Approximately 2,600 references were obtained from the initial literature search for 1,2,4-TMB, 1,2,3-TMB, and 1,3,5-TMB, including references retrieved from specific literature searches necessary for the interpretation of TMB-induced health effects (e.g., literature on specific modes of action, physiologically based pharmacokinetic [PBPK] analysis), or including references identified from the primary literature. From this full list of references, there were 143 references that were selected for inclusion in the Toxicological Review. Preamble references are included in these counts unless they were cited in the Toxicological Review.

The initial literature search was augmented with an updated literature search to identify references published since December 2011 that could provide information relevant to the assessment. This literature search identified approximately 960 references, 5 of which were included in the assessment. This literature search was conducted using similar search terms as the initial literature search, but did not use the EBSCO Discovery Service and instead searched Toxicology Literature Online (TOXLINE), National Library of Medicine (NLM), and Web of Science (WOS) databases separately. This difference in search methodology resulted in proportionally more references being identified over a shorter time span (2011–2016 versus pre-2011), but fewer being deemed relevant to the assessment (5/962 versus 145/2,611). During the external peer review process, the Chemical Assessment Advisory Committee (CAAC) recommended the inclusion of 15 studies, which were added to the assessment. The CAAC further recommended including studies investigating the health effects due to exposure of compounds structurally similar to TMBs (e.g., toluene, xylene, etc.). In order to identify relevant studies, EPA conducted a targeted literature search for review articles in PubMed using the following query: (("styrene"[All Fields] or toluene[All Fields] or xylene[All Fields] or ethyltoluene[All Fields]) and (neurotoxicity[All Fields] or "respiratory toxicity"[All Fields] or "hematological toxicity"[All Fields] or (("developmental"[All Fields] or "respiratory"[All Fields]) and "toxicity"[All Fields]))) and Review[Publication Type]. A total of 69 review articles were identified. Of the identified review articles, 14 were cited in the assessment, and 6 primary references identified from the review articles were also included. In total, 185 references were cited in the assessment when considering references identified in the initial, update, and related compound literature searches in addition to the specific references recommended for inclusion by the CAAC. The references that are cited in the document, as well as those that were considered but not included in the *Toxicological Review of Trimethylbenzenes*, can

be found within the Health and Environmental Research Online (HERO) website: https://hero.epa.gov/hero/index.cfm/project/page/project_id/2375. This site contains HERO links to lists of references, including bibliographic information and abstracts, which were considered for inclusion in the *Toxicological Review of Trimethylbenzenes*.




Note: Some references may provide information on more than one topic, and therefore, may be included in more than one study type. Accordingly, the sum of the references for subcategories of studies is not expected to equal the number of references for the larger category. Preamble references are not counted unless actually cited in the Toxicological Review.

**Figure LS-1. Literature search and study selection strategy for TMBs.**

# 1. HAZARD IDENTIFICATION

This Hazard Identification section critically reviews the publicly available studies on the three isomers of trimethylbenzene (TMBs) (i.e., 1,2,3-TMB, 1,2,4-TMB, and 1,3,5-TMB) in order to describe their toxicokinetics, identify their adverse health effects, and characterize whether these compounds pose organ/system-specific hazards to humans. In evaluating this isomer-specific toxicity databases, the most consistently observed effect is neurotoxicity, and as such, most of the hazard discussion is focused on effects in the nervous system. Neurotoxicity is observed in human populations exposed to solvent mixtures containing TMBs, and in laboratory animals exposed to single TMB isomers for acute, short-term, and subchronic durations. In addition to neurotoxicity, TMB isomers are observed to elicit effects in the respiratory and hematological systems, and are reported to result in adverse effects in pregnant animals and the developing fetus. Each of these health effects, in addition to sections describing the general toxicity effects and carcinogenicity of TMB isomers, are included in their own separate section, which includes discussions of the various toxicities observed, as well as evidence tables and exposure-response arrays that further summarize the data (Sections 1.2.1–1.2.6). Following each organ-specific section, mode-of-action and summary sections are included to present evidence syntheses and discuss possible modes of action and their potential relevance in hazard determinations. A general paucity of chemical-specific information exists regarding possible modes of action, but some insights can be gained by considering information on related compounds and/or mixtures containing TMB isomers. Section 1.2.7 provides a summary of how the three TMB isomers are similar to one another regarding observed toxicities. This determination that the three TMB isomers are observed to result in largely similar toxicological profiles, especially neurotoxicity effects, is of particular importance for subsequent dose-response analyses. The overall weight of evidence for individual noncancer and cancer endpoints is synthesized in Section 1.3.1.

In addition to isomer-specific animal toxicity studies, four studies that investigated the toxicity in rodent species following exposure to the C9 aromatic fraction are included. The C9 aromatic fraction is a mixture of volatile organic compounds (VOCs) containing an aromatic ring and nine total carbons. Although the majority of the C9 fraction is composed of TMB isomers (>50%), these studies generally report low levels of C9-induced toxicity. This discrepancy between toxicity profiles of individual TMB isomers and the C9 fraction is also discussed.

# 1.1. OVERVIEW OF THE TOXICOKINETICS OF TMBs

## 1.1.1. Toxicokinetics of TMB isomers

In the existing toxicokinetic database for 1,2,3-TMB, 1,2,4-TMB, and 1,3,5-TMB, important similarities have been observed in the chemical properties and absorption, distribution, metabolism, and excretion (ADME) profiles for these isomers in animals and humans, although some important differences also exist. A summary of these comparisons across individual TMB isomers is presented in Table 1-1; for comparison of metabolic schemes, see Figures C-1 through C-3.

All three isomers have very similar log $K_{ow}$ values (3.42–3.78), but a wider distribution of Henry's law constants (4.36–8.77 × $10^{-3}$ atm × $m^3$/mol). The isomers' blood:air partition coefficients reported for humans and rats in the literature are similar: 43.0 and 55.7, respectively, for 1,3,5-TMB, 66.5 and 62.6, respectively, for 1,2,3-TMB, and 59.1 and 57.7, respectively, for 1,2,4-TMB (Meulenberg and Vijverberg, 2000). This gives an indication that the three isomers would partition into the blood in a similar fashion. Supporting this is the observation that 1,2,4-TMB and 1,3,5-TMB absorb equally into the bloodstream of exposed humans (6.5 and 6.2 μM, respectively), although the absorption for 1,2,3-TMB was observed to be higher (7.3 μM) (Järnberg et al., 1998, 1997a; Järnberg et al., 1996). The net respiratory uptake of 1,2,3-TMB, 1,2,4-TMB, and 1,3,5-TMB was similar among humans (48–60%), and the respiratory uptake for 1,2,4-TMB was also similar across humans and rats (50–60%) (Järnberg et al., 1996; Dahl et al., 1988). Although no data exist regarding the distribution of TMB isomers in humans, experimentally-derived, tissue-specific partition coefficients were similar for all three isomers across a number of systems (Meulenberg and Vijverberg, 2000), strongly suggesting that the individual isomers can be expected to distribute similarly to these various systems. Distribution of 1,2,4-TMB, 1,2,3-TMB, and 1,3,5-TMB throughout the body is qualitatively similar in animals, although it appears that there are some quantitative differences. Liver concentrations for 1,2,4-TMB are greater than those for 1,3,5-TMB after both acute and short-term inhalation exposures at all concentrations, whereas 1,2,4-TMB liver concentrations were only greater than 1,2,3-TMB concentrations at the mid- and high doses (Świercz et al., 2016; Swiercz et al., 2006; Swiercz et al., 2003; Swiercz et al., 2002). Although 1,2,4-TMB was observed to distribute to the brain (Swiercz et al., 2003; Eide and Zahlsen, 1996), distribution of 1,3,5-TMB or 1,2,3-TMB to the brain was not experimentally measured in any study. However, the predicted brain:air partition coefficient were similar between 1,2,4-TMB, 1,2,3-TMB, and 1,3,5-TMB for both humans (206, 220, and 199, respectively) and rats (552, 591, and 535, respectively) (Meulenberg and Vijverberg, 2000). This strongly suggests that 1,2,4-TMB, 1,2,3-TMB, and 1,3,5-TMB can be expected to distribute similarly to the brain in both humans and rats. The observation that organ concentrations are lower following repeated exposures to TMB isomers compared to acute exposures is mostly likely due to induction of metabolizing enzymes at higher exposure concentrations. This hypothesis is supported by observation of cytochrome P-450

(CYP450) enzyme induction in the livers, kidneys, and lungs of rats exposed to 1,2,4-TMB (Pyykko, 1980).

**Table 1-1. Toxicokinetic similarities between TMB isomers**

| Toxicokinetic or metabolic parameter | | Species | TMB isomer ranking[a] |
|---|---|---|---|
| **Absorption** | | | |
| Capillary blood concentration | | Humans | 1,2,3-TMB > 1,2,4-TMB ≈ 1,3,5-TMB |
| Respiratory uptake | | Humans | 1,2,4-TMB > 1,3,5-TMB > 1,2,3-TMB |
| Blood:air partition coefficient | | Humans | 1,2,4-TMB ≈ 1,2,3-TMB > 1,3,5-TMB |
| Blood:air partition coefficient | | Rats | 1,2,4-TMB ≈ 1,2,3-TMB ≈ 1,3,5-TMB |
| **Distribution** | | | |
| Liver concentration (4-wk exposure) | 123 mg/m$^3$<br>492 mg/m$^3$<br>1,230 mg/m$^3$ | Rats | 1,2,3-TMB > 1,2,4-TMB ≈ 1,3,5-TMB<br>1,2,4-TMB ≈ 1,2,3-TMB ≈ 1,3,5-TMB<br>1,2,4-TMB > 1,2,3-TMB ≈ 1,3,5-TMB |
| Kidney concentration (4-wk exposure) | 123 mg/m$^3$<br>492 mg/m$^3$<br>1,230 mg/m$^3$ | Rats | 1,2,3-TMB > 1,3,5-TMB<br>1,3,5-TMB > 1,2,3-TMB<br>1,2,3-TMB ≈ 1,3,5-TMB |
| Lung concentration (4-wk exposure) | 123 mg/m$^3$<br>492 mg/m$^3$<br>1,230 mg/m$^3$ | Rats | 1,2,3-TMB > 1,2,4-TMB ≈ 1,3,5-TMB<br>1,2,4-TMB > 1,2,3-TMB ≈ 1,3,5-TMB<br>1,2,4-TMB ≈ 1,2,3-TMB > 1,3,5-TMB |
| Brain:air partition coefficient | | Humans | 1,2,4-TMB ≈ 1,2,3-TMB ≈ 1,3,5-TMB |
| Brain:air partition coefficient | | Rats | 1,2,4-TMB ≈ 1,2,3-TMB ≈ 1,3,5-TMB |
| **Metabolism** | | | |
| Urinary hippuric acids (% inhaled TMB dose, 25 ppm) | | Humans | 1,2,4-TMB > 1,2,3-TMB >> 1,3,5-TMB |
| Urinary hippuric acids (% oral TMB dose, 1.2 g/kg) | | Rats | 1,3,5-TMB >> 1,2,4-TMB > 1,2,3-TMB |
| **Excretion** | | | |
| Total blood clearance | | Humans | 1,3,5-TMB > 1,2,4-TMB ≈ 1,2,3-TMB |
| Terminal half-life of elimination | | Humans | 1,3,5-TMB > 1,2,4-TMB ≈ 1,2,3-TMB |
| Terminal half-life of elimination (123 mg/m$^3$) | | Rats | 1,2,4-TMB ≈ 1,2,3-TMB ≈ 1,3,5-TMB |
| Terminal half-life of elimination (1,230 mg/m$^3$) | | Rats | 1,2,4-TMB > 1,2,3-TMB ≈ 1,3,5-TMB |

[a] Greater than, lesser than, or approximation symbols are intended to provide a general, qualitative sense of the comparative differences between isomers. Refer to study summary tables in Appendix C for exact values.

All three TMB isomers were observed to primarily metabolize to benzoic and hippuric acids in humans and rats (Järnberg et al., 1996; Huo et al., 1989; Mikulski and Wiglusz, 1975), although the amounts of inhaled TMB recovered as hippuric acid metabolites following exposure to 1,2,3-TMB, 1,2,4-TMB, or 1,3,5-TMB were dissimilar in humans (11, 22, and 3%, respectively) and rats (10, 24–38, and 59%, respectively) (Järnberg et al., 1996; Mikulski and Wiglusz, 1975). Greater

amounts of urinary benzoic acid and hippuric acid metabolites (73%) were observed after exposure to higher amounts of 1,3,5-TMB (up to 30.5 ppm) for 8 hours (Kostrzewski et al., 1997; Kostrewski and Wiaderna-Brycht, 1995). Other terminal metabolites included mercapturic acids (~14–19% total dose), phenols (~12% total dose), and glucuronides and sulphuric acid conjugates (4–9% total dose) for 1,2,4-TMB; mercapturic acids (~5% total dose), phenols (<1–8% total dose), and glucuronides and sulphuric acid conjugates (8–15% total dose) for 1,2,3-TMB; and phenols (~4–8% total dose) and glucuronides and sulphuric acid conjugates (~5–9% total dose) for 1,3,5-TMB (Tsujimoto et al., 2005; Tsujimoto et al., 2000, 1999; Huo et al., 1989; Wiglusz, 1979; Mikulski and Wiglusz, 1975). Although no evidence exists to definitely identify the toxic moiety, it is assumed that the parent compound and not any of the multiple metabolites of TMBs are the toxic moiety of interest.

In humans, the half-lives of elimination from blood were observed to be similar for all isomers in the first three phases of elimination: 1,2,4-TMB (1.3 ± 0.8 minutes, 21 ± 5 minutes, 3.6 ± 1.1 hours), 1,2,3-TMB (1.5 ± 0.9 minutes, 24 ± 9 minutes, 4.7 ± 1.6 hours), and 1,3,5-TMB (1.7 ± 0.8 minutes, 27 ± 5 minutes, 4.9 ± 1.4 hours) (Järnberg et al., 1996). The half-life of elimination for 1,3,5-TMB in the last and longest phase is much greater than those for 1,2,4-TMB or 1,2,3-TMB (120 ± 41 versus 87 ± 27 and 78 ± 22 hours, respectively). Urinary excretion of unchanged parent compound was extremely low (<0.002%) for all three isomers (Janasik et al., 2008; Järnberg et al., 1997b). The difference observed in half-lives between the three isomers in the last elimination phase may be due to small sample sizes and difficulties in measuring slow elimination phases rather than a true difference in half-lives. All three isomers were eliminated via exhalation: 20–37% of the absorbed dose of 1,2,4-TMB, 1,2,3-TMB, or 1,3,5-TMB was eliminated via exhalation during exposure to 123 mg/m$^3$ (25 ppm) for 2 hours (Järnberg et al., 1996).

Following exposure of rats to 25 ppm (123 mg/m$^3$) 1,2,4-TMB, 1,2,3-TMB, or 1,3,5-TMB for 6 hours, the terminal half-life of elimination of 1,3,5-TMB from the blood (2.7 hours) was shorter than that for 1,2,4-TMB (3.6 hours) or 1,2,3-TMB (3.1 hours) (Świercz et al., 2016; Swiercz et al., 2006; Swiercz et al., 2002). As dose increased, the half-lives for elimination from blood following single exposures to 1,2,4-TMB (17.3 hours) became much longer than those for 1,3,5-TMB (4.1 hours) or 1,2,3-TMB (5.3 hours). In repeated-dose experiments (4 weeks), the terminal half-lives of elimination of TMB isomers in venous blood were similar for 1,2,4-TMB and 1,2,3-TMB (9.9 and 8.0 hours, respectively), but larger than that of 1,3,5-TMB (4.6 hours) (Świercz et al., 2016; Swiercz et al., 2006; Swiercz et al., 2003; Swiercz et al., 2002).

For a full discussion of the toxicokinetics of 1,2,3-TMB, 1,2,4-TMB, and 1,3,5-TMB, see Appendix C, Section C.1.

### 1.1.2. Description of Toxicokinetic Models

There were three deterministic physiologically-based pharmacokinetic (PBPK) models identified in the literature for TMB isomers. Järnberg and Johanson (1999) developed a PBPK model for inhalation of 1,2,4-TMB in humans in order to investigate how various factors (work load,

exposure level, fluctuating exposure) influence potential biomarkers of exposure (end-of-shift and prior-to-shift concentrations of parent compound in blood and metabolites in urine). There was no animal component to this PBPK model and it was not parameterized for other TMB isomers. Emond and Krishnan (2006) developed a PBPK model not for any TMB isomer specifically, but to test whether a model could be developed for highly lipophilic VOCs (HLVOCs) using the neutral lipid equivalent (NLE) content of tissues and blood as the basis. Lastly, Hissink et al. (2007) developed a PBPK model to characterize internal exposure following white spirit inhalation. This model contained both a rat and a human component and was constructed to be able to predict levels of 1,2,4-TMB and *n*-decane in the blood and brain following exposure to white spirit.

All three of the available 1,2,4-TMB PBPK models were evaluated for potential use in this assessment. Of the three deterministic PBPK models available for 1,2,4-TMB (Hissink et al., 2007; Emond and Krishnan, 2006; Järnberg and Johanson, 1999), the Hissink et al. (2007) model was chosen for this assessment because it was the only published 1,2,4-TMB model that included parameterization for both rats and humans, the model code was available, and the model adequately predicted experimental data in the dose range of concern. The Hissink et al. (2007) model was thoroughly evaluated, including a detailed computer code analysis. Full details on model verification, parameterization, optimization, and validation (including all analyses to confirm published model outputs), discussions of the uncertainties in model structure and choice of dose metric, and sensitivity analyses are provided in Appendix C, Section C.3.3.

## 1.2. SYNTHESIS OF EVIDENCE

### 1.2.1. Neurological Effects

There is evidence in humans and animals that inhalation exposure to TMBs induces neurotoxic effects. The human evidence comes from occupational studies involving complex VOC mixtures that include TMBs; thus, effects cannot be attributed to any TMB isomer specifically. Prevalence rates of neuropsychological symptoms increased with exposure duration in dockyard painters, with symptoms related to motor coordination exhibiting the strongest association (Chen et al., 1999). Similarly, significant associations between exposure and impaired performance in short-term memory (symbol digit substitution), motor speed/coordination (finger tapping), and peripheral nerve function tests were observed in other studies of shipyard painters exposed to mixtures possibly containing TMBs (isomers were not specified) and other solvents (Lee et al., 2005; Ruijten et al., 1994). Other neuropsychological symptoms (mood changes, equilibrium complaints, and sleep disturbances) were also reported by Ruijten et al. (1994). Detrimental neuropsychological effects (memory problems, dizziness, hand tremble) have also been reported in paint factory workers exposed to multiple unspecified solvents; working in jobs with an ostensible higher exposure to solvents (production versus packaging) was observed to be among the strongest predictors of symptoms (El Hamid Hassan et al., 2013). A significant, positive association between

exposure symptoms (e.g., abnormal fatigue) and 1,2,4-TMB exposure, but not exposure to lower levels of 1,2,3-TMB or 1,3,5-TMB, was reported in asphalt workers (Norseth et al., 1991). Nervousness, tension, headaches, vertigo, and anxiety were reported in paint shop workers exposed to 49–295 mg/m$^3$ of a solvent mixture containing 50% 1,2,4-TMB, 30% 1,3,5-TMB, and unspecified amounts of 1,2,3-TMB (listed as possibly present) (Bättig et al. (1956), as reviewed by MOE (2006) and Bättig et al. (1958)).

Additional evidence suggests damage or dysfunction of the inner ear and increased occurrence of vertigo following exposure to TMBs and other organic solvents in paint and varnish factories and histology laboratories (Juárez-Pérez et al., 2014; Fuente et al., 2013; Sulkowski et al., 2002). However, an analysis using naphthalene as a marker for jet propulsion fuel 8 (JP-8) (which contains TMB isomers among multiple other aliphatic and aromatic solvents) did not indicate that exposure to complex solvent mixtures resulted in increased postural sway (Maule et al., 2013). Similar to suggestive evidence of visual impairment following exposure to complex solvent mixtures (i.e., altered color vision and contrast in exposed furniture factory workers (Gong et al., 2003) and increased latencies for visual evoked potentials [VEPs] in gasoline-exposed workers (Pratt et al., 2000)), increased reaction time was significantly and consistently associated with exposure in controlled, acute volunteer studies in which adults were exposed to mixtures containing 1,2,4-TMB (Lammers et al., 2007), although it is unclear whether 1,2,4-TMB or other constituents within the mixtures were responsible for the observed effects. However, in another volunteer study in which participants were exposed to aromatic or dearomatized white spirit for 4 hours, neurobehavioral impairments were either weakly or inconsistently associated with exposure (Juran et al., 2014). Uptake of TMBs was reported in volunteers exposed for 2 hours to either 300 mg/m$^3$ white spirit (corresponding to 11 mg/m$^3$ 1,2,4-TMB); 11 or 123 mg/m$^3$ 1,2,4-TMB; 123 mg/m$^3$ 1,2,3-TMB; or 123 mg/m$^3$ 1,3,5-TMB. However, effects on the central nervous system (CNS) were based on measures of overt CNS depression (heart rate and pulmonary ventilation) and a subjective rating of CNS symptoms (i.e., headache, fatigue, nausea, dizziness, and intoxication) (Järnberg et al., 1997a; Järnberg et al., 1996). For full details of the epidemiologic and controlled human exposures studies (including human subjects research ethics procedures), see individual study summary tables in Appendix C.

In two studies examining the toxicokinetics of TMBs following controlled human exposures to 5–150 mg/m$^3$ 1,2,3-TMB, 1,2,4-TMB, or 1,3,5-TMB, no neurological abnormalities in routine clinical examinations were reported following exposure, although neither results data nor details regarding the specific tests performed were provided (Kostrzewski et al., 1997; Kostrewski and Wiaderna-Brycht, 1995). Studies identifying an association between occupational exposure to TMB isomers and neurological effects are limited due to an inability to attribute effects due to 1,2,3-TMB, 1,2,4-TMB, or 1,3,5-TMB individually versus those due to the other isomers or additional constituents within the mixture. The studies detailing controlled exposures to volunteers are also limited for evaluating neurotoxicity to TMBs due to a lack of methods to adequately assess CNS

function, lack of no-exposure controls, short exposure duration, and exposure of individual subjects to different concentrations of TMB isomers.

In animals, there is consistent evidence of neurotoxicity following inhalation exposure, and to a lesser extent following oral exposure, to either 1,2,3-TMB, 1,2,4-TMB, or 1,3,5-TMB; a summary of the evidence pertaining to neurotoxic effects for TMBs is shown in Tables 1-2 and 1-3 for inhalation and oral exposures, respectively. This information is presented graphically in Figure 1-1.

***Pain sensitivity***

No data on pain-related behaviors were identified from studies of humans exposed to individual TMB isomers or mixtures containing TMBs. Decreased pain sensitivity has been observed following inhalation exposure to TMBs in multiple studies conducted in male Wistar rats (Table 1-2; Figure 1-1). To test pain responses following TMB exposure, animal studies have employed the hot plate test. In this test, animals are exposed to a thermal stimulus by placing them on a copper plate heated to 54.5°C in order to determine pain sensitivity (Ankier, 1974). The latency for the animals is recorded as the time it takes for the tested animals to remove their paws from the heated plate and lick their paws, with longer latencies reflecting decreased pain sensitivity. In order to investigate potential TMB-induced sensitizing and/or latent effects, the short-term exposure studies introduced an additional challenge to the testing paradigm in the form of a footshock. By incorporating the footshock, which itself decreases pain sensitivity, before testing hot plate responses, the short-term exposure studies were able to investigate potentially more subtle TMB-induced neurotoxic effects long after the termination of exposure (testing began >50 days post-exposure). Specifically, the footshock challenge introduces a stressor on nervous system processes related to pain perception (essentially straining the capabilities of this system either through desensitization of sensory pain receptors or damage to the peripheral nerves), which appears to be capable of unmasking latent nervous system effects that may persist for weeks after TMB isomer exposure.

Decreases in pain sensitivity have been observed at concentrations ≥492 or ≥123 mg/m$^3$ following subchronic exposure to 1,2,4-TMB or 1,2,3-TMB, respectively (Korsak and Rydzyński, 1996). Decreased pain sensitivity after a footshock challenge was observed at concentrations ≥492 mg/m$^3$ following short-term exposure to 1,2,4-TMB (Gralewicz and Wiaderna, 2001; Gralewicz et al., 1997b), 1,3,5-TMB (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001), or 1,2,3-TMB (Wiaderna et al., 1998), although changes were not observed at 492 mg/m$^3$ 1,2,3-TMB (latencies 75% longer than controls were not statistically significant) in another short-term exposure study (Gralewicz and Wiaderna, 2001). No statistically significant decreases in pain sensitivity were observed 24 hours after exposure in rats exposed to concentrations of the C9 fraction up to 1,500 ppm for up to 13 weeks (approximately 4,059 mg/m$^3$ TMB isomers) (Douglas et al., 1993). There was a statistically significant increase in thermal response time in C9-exposed groups when the effect was measured immediately prior to the exposure period; however, this was most likely due to an unusually low control group response at this time point. Specifically

regarding pain sensitivity measured immediately after exposure, effects were more pronounced following subchronic exposure as compared to acute exposure (e.g., for 1,2,4-TMB, a 50% response, or 30-second latency, required levels nearly 5-fold higher: 5,682 versus <1,230 mg/m$^3$, with exposure for 4 hours as compared to for 13 weeks) (Korsak and Rydzyński, 1996).

In the subchronic exposure study (Korsak and Rydzyński, 1996), inhalation of 1,2,4-TMB or 1,2,3-TMB resulted in reduced pain sensitivity, which occurred in a concentration-dependent manner. In short-term exposure studies that examined a range of concentrations (Wiaderna et al., 2002, 1998; Gralewicz et al., 1997b), decreases in pain sensitivity after footshock challenge following exposure to TMB isomers were non-monotonic, with exposure to 1,230 mg/m$^3$ TMB usually resulting in no response or a substantially reduced response as compared to lower TMB concentrations (e.g., 492 mg/m$^3$). Additionally, no effect on pain sensitivity was observed in rats exposed to the C9 fraction. Differences in experimental design (discussed below) or mechanism of action may account for the lack of monotonicity in TMB short-term exposure studies and no effect in the C9 study, in contrast to the observations in Korsak and Rydzyński (1996). Similar to the subchronic study, acute exposures to 1,2,3-TMB, 1,2,4-TMB, 1,3,5-TMB, or Farbasol (a solvent mixture containing 44% TMB isomers) induced concentration-dependent decreases in pain sensitivity, with $EC_{50}$ values of 4,172, 5,682, 5,963, or 6,589 mg/m$^3$, respectively, for increased latency to paw-lick compared to controls (Korsak et al., 1999; Korsak and Rydzyński, 1996; Korsak et al., 1995).

The decreases in pain sensitivity measured in the subchronic and acute TMB-only studies were observed immediately after exposure (Korsak and Rydzyński, 1996; Korsak et al., 1995), with no statistically significant effects persisting 2 weeks after subchronic exposures were terminated (i.e., increases in latency of 95 or 78% greater than controls were reduced to 12 or 13% greater than controls at 1,230 mg/m$^3$ for 1,2,4- or 1,2,3-TMB, respectively) (Korsak and Rydzyński, 1996; Korsak et al., 1995). Similarly, short-term TMB exposure without the footshock challenge did not result in statistically significant effects on pain sensitivity in the hot plate test several weeks after exposures had ended, although latencies were increased up to 200%. In contrast, performance in the hot plate test after footshock challenge was significantly impaired following short-term exposure to the TMB isomers when tested 51 days after exposure (Wiaderna et al., 1998) (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Gralewicz et al., 1997b), indicating a persistence of these pain sensitivity-related effects. The footshock data suggest that, following short-term exposure, TMB isomers caused a long-term (potentially permanent) reduction in the ability of the nervous system to respond to, and/or recover from, the stress-induced analgesia caused by footshock.

The addition of a footshock challenge to the hot plate tests following short-term (i.e., 4-week), inhalation exposure to TMB isomers makes these experiments somewhat distinct from those performed following subchronic exposure, as the footshock challenge can elicit a cognitive response from the animals in later hot plate test trials (see below) (Wiaderna et al., 2002; Gralewicz

and Wiaderna, 2001; Wiaderna et al., 1998; Gralewicz et al., 1997b). In the short-term studies (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998; Gralewicz et al., 1997b), treatment-related, statistically significant changes at ≥492 mg/m$^3$ 1,2,3-TMB, 1,2,4-TMB, or 1,3,5-TMB were observed 24 hours after rats were given a footshock; no consistent, significant effects at any concentration were observed immediately following footshock, when pain sensitivity is suppressed by footshock. Additionally, no statistically significant effects were observed prior to footshock at 50 days post-exposure; studies did tend to observe increases in latency in non-shocked rats, which were not statistically significant at ≥492 mg/m$^3$ 1,2,4-TMB (up to 206% longer than controls), 1,3,5-TMB (up to 215% longer than controls), or 1,2,3-TMB (up to 95% longer than controls), but these responses were highly variable and not consistently observed across studies. As footshock alone is known to cause transient reductions in pain sensitivity, these findings suggest that inhalation exposure to TMBs prolongs footshock-induced reductions in pain sensitivity. However, although a lengthening of the footshock-induced decrease in pain sensitivity by TMB exposure is the most likely reason for the observed effects, and, accordingly, these responses are discussed in this context herein, this is not the only possible explanation. It is also possible that cognitive effects resulting from TMB exposure might contribute to the responses observed 24 hours after footshock. Specifically, control groups may better associate the hot plate environment with the previously-applied aversive stimulus and more quickly withdraw their paws than their TMB-exposed counterparts that may exhibit a decreased fear response or shorter retention of that fear-associated memory. Alternatively, since this test paradigm can cause the hot plate test apparatus to become associated with the effects of footshock, inducing stress-related responses in the shocked animal such that subsequent exposure to the hot plate test apparatus alone can reduce sensitivity to pain (possibly via the release of endogenous opioids), prior TMB exposure could amplify this effect. From the data available, the relative contribution(s) of these behaviors to the observed effects cannot be easily distinguished. Despite the possible overlap between contributing neurological processes in this test paradigm, these observations are still regarded as significant and adverse, and indicate a persistence of neurological effects long after TMB exposures have ceased.

Differences in study design may also partially explain the difference in results observed in the subchronic studies, in which statistically significant decreases in pain sensitivity were observed when measured immediately after termination of exposure (Korsak and Rydzyński, 1996), but not when measured 24 hours after exposure (Douglas et al., 1993) (information provided in written comments submitted during public comment period). As stated above, decreased pain sensitivity was mostly reversible when measured 2 weeks post-exposure in a subchronic exposure study (Korsak and Rydzyński, 1996) and 24 hours after the termination of exposure (Douglas et al., 1993). Additionally, when the endpoint was measured up to 50 days after the termination of short-term exposures (i.e., pre-footshock), there was no observable effect (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998; Gralewicz et al., 1997b). However, when animals were subjected to an environmental stimulus (i.e., footshock), decreased pain sensitivity

was observed in exposed animals, demonstrating a persistence of effect. While the U.S. Environmental Protection Agency (EPA) *Guidelines for Neurotoxicity Risk Assessment* (U.S. EPA, 1998) note that effects that are reversible in minutes, hours, or days after the end of exposure and appear to be associated with the pharmacokinetics of the agent and its presence in the body may be of less concern than effects that persist for longer periods of time, they also note that it is important to consider that effects that may seem reversible may re-appear later or be predictive of later adverse outcomes. While the Douglas et al. (1993) study appears to present differing results compared to the subchronic and short-term exposure studies (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998; Gralewicz et al., 1997b; Korsak and Rydzyński, 1996), it actually comports with the subchronic and short-term studies when considering the potential time-course of the decreased pain sensitivity endpoint. Decreased pain sensitivity is clearly observed immediately after termination of subchronic exposure to either 1,2,4-TMB or 1,2,3-TMB (Korsak and Rydzyński, 1996), and to a lesser extent following acute exposure; this effect then becomes undetectable when measured at time points further from the end of the exposure period (24 hours: Douglas et al. (1993); 2 weeks: Korsak and Rydzyński (1996); and 50 days: Gralewicz et al. (1997b) and Wiaderna et al. (1998)), and then becomes evident again as a latent effect following challenge with an environmental stimulus (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998; Gralewicz et al., 1997b). Additional differences in study design that may explain some discrepancies in observed effects include the fact that Douglas et al. (1993) used the C9 aromatic fraction as the test agent and not a single TMB isomer or mix thereof, and how the hot plate test was administered Douglas et al. (1993) did not provide exact details on the hot plate temperature. It is not currently known how constituents of the C9 fraction, including TMBs, would be absorbed and distributed throughout the body after exposure, as no toxicokinetic studies are available that have investigated this topic. Therefore, it is not known whether TMB isomers in the C9 fraction partition to the target organ system (i.e., the nervous system) in a similar pattern as TMB isomers when used as the sole exposure agent.

Substantial differences in study design between short-term and subchronic exposure studies, and a lack of knowledge regarding the specific mode(s) of action, make it impossible to distinguish the particular aspects of the pain sensitivity phenotype that appear to be latent and only manifest with an environmental challenge from those that appear to be at least partially reversible. Regardless, the ability of male Wistar rats to respond to a thermal stimulus in the hot plate test was consistently impaired following inhalation exposure to individual TMB isomers. The overall database indicates that individual TMB isomers are similar in their capacity to decrease pain sensitivity following inhalation exposure (Table 1-2; Figure 1-1). Pain sensitivity was not examined following oral exposure.

***Neuromuscular function and coordination***

Human exposures to solvent mixtures containing 1,2,4-TMB (Lammers et al., 2007), multiple TMB isomers (Bättig et al. (1956), as reviewed by MOE (2006) and (Lee et al. (2005);

Sulkowski et al. (2002); Bättig et al. (1958)), or complex solvent mixtures (El Hamid Hassan et al., 2013; Ruijten et al., 1994) result in effects that suggest alterations to neuromuscular function and balance, including increased reaction time, increased hand tremble, decreased hand eye coordination, and vertigo. Animal studies using rotarod performance, which tests motor coordination, balance, and overall neuromuscular function, indicate that inhalation of TMB isomers can affect neuromuscular system function (Table 1-2; Figure 1-1). Significant decreases in rotarod performance were observed at 1,230 mg/m$^3$ 1,2,4-TMB (40% response) and ≥492 mg/m$^3$ 1,2,3-TMB (50–70% response) when tested immediately after exposure for 13 weeks (Korsak and Rydzyński, 1996); an exposure duration-dependency for this effect was observed, with less robust, but statistically significant, decreases in performance also reported at 1,230 mg/m$^3$ after 4 (40 and 30% response) or 8 (60 and 40% response) weeks of exposure to 1,2,3-TMB or 1,2,4-TMB, respectively. This impaired function was still evident at 2 weeks post-exposure, indicating a persistence of this effect. Specifically, failures in 70 and 40% of animals after 13 weeks of exposure to 1,230 mg/m$^3$ 1,2,3-TMB and 1,2,4-TMB, respectively (compared to 0% of animals in control groups at any time), were 50 and 30% at 2 weeks post-exposure, although 30% failure at 15 weeks for 1,2,4-TMB was no longer significantly different from controls (note: statistical comparisons did not appear to include a repeated measures component and comparisons to the 13-week time-point were not performed). The observations of substantial decrements in rotarod performance are interpreted as a biologically relevant responses in light of the lack of failures in controls and the similarities in response magnitude across isomers. Inhalation studies of acute TMB exposure support this observation. Effects such as loss of reflexes and righting responses have been observed following acute inhalation exposure to 1,250–45,000 mg/m$^3$ 1,2,4-TMB (MOE, 2007, 2006; Henderson, 2001; Cameron et al., 1938). Similarly, acute exposure to 1,2,3-TMB, 1,2,4-TMB, 1,3,5-TMB, or Farbasol resulted in decreased performance in rotarod tests immediately following exposure, with $EC_{50}$ values of 3,779, 4,693, 4,738, or 5,497 mg/m$^3$, (Korsak et al., 1999; Korsak and Rydzyński, 1996; Korsak et al., 1995). The potential for reversibility of effects following acute exposure has not been tested. These results indicate that 1,2,4-TMB and 1,3,5-TMB are similar in their ability to impair neuromuscular function, balance, and coordination, while 1,2,3-TMB exposure may elicit effects at lower concentrations compared to the other two isomers. Other tests of neuromuscular function and/or coordination (grip strength, hindfoot splay) were not affected at any exposure concentration (up to 1,500 ppm C9; approximately 4,059 mg/m$^3$ TMB isomers) in exposed rats (Douglas et al., 1993). No studies evaluating oral exposure to TMB isomers address this endpoint.

The neurobehavioral tests administered (i.e., hot plate and rotarod) in the subchronic and acute exposure studies by Korsak and Rydzyński (1996) and Korsak et al. (1995) appear to have been conducted on the same days; however, it is unclear whether the tests were performed sequentially in the same cohorts of animals. Performing the hot plate test immediately following the rotarod test could introduce a potential confounder, as shock alone (such as that used as

negative reinforcement following rotarod failure, see Table C-28, Appendix C) can cause reductions in pain sensitivity. Thus, if the tests were performed sequentially in the same animals, TMB-exposed animals failing more often in the rotarod test may exhibit increases in paw-lick latency unrelated to treatment, as compared to controls receiving less shock reinforcement. However, the observations by Korsak and Rydzyński (1996) and Korsak et al. (1995) are supported by 2–3-fold increases in latency to paw-lick that, although not statistically significant but possibly biologically relevant given the magnitude and consistency of response across isomers, were observed 50 days after termination of short-term exposures to 492 mg/m$^3$ 1,2,3-TMB, 1,2,4-TMB, or 1,3,5-TMB (Gralewicz and Wiaderna, 2001); increases of this magnitude were not present in the studies evaluating multiple concentrations of the isomers (Wiaderna et al., 2002, 1998; Gralewicz et al., 1997b).

***Motor function and/or anxiety***

Effects in open field testing have been consistently reported in oral and inhalation studies of exposure to 1,2,4-TMB and 1,3,5-TMB, but not 1,2,3-TMB or the C9 fraction, in male rats (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998; Gralewicz et al., 1997b) (Table 1-2; Figure 1-1); however, open field locomotion following injections with the stimulant, amphetamine, was amplified by prior 1,2,3-TMB exposure, but not by prior 1,2,4-TMB exposure (Lutz et al., 2010), suggesting a more complicated pattern to these effects. As noted in the U.S. EPA (1998) neurotoxicity guidelines, motor activity tests are typically designed to be of sufficient duration to allow control animals to acclimate to the novel environment and reduce their activity during the latter stages of testing. In contrast, the brief open field tests employed in the current studies (most studies observed animals for 5 or 10 minutes) measure behaviors and exploratory locomotion, which are strongly influenced by initial anxiety responses due to the open spaces and bright light, as well as by potential changes to motor system function (Carola et al., 2002; Broadhurst, 1969); to a lesser extent, factors other than anxiety and motor function (e.g., interpretation of olfactory or visual cues) may also contribute. As no studies assessed parameters that could inform to what extent anxiety-related responses may have been affected in these tests (e.g., increased time or activity in central regions of the field, as compared to peripheral regions bordering the walls, is indicative of decreased anxiety-related responses), EPA has concluded that decreased anxiety and/or increased motor function are the most likely explanations for the TMB-induced increases in activity in these brief open field tests.

Decreased anxiety and/or increased motor function at ≥492 mg/m$^3$ 1,2,4-TMB or 1,3,5-TMB has been reported in short-term exposure studies several weeks after exposures ceased, as evidenced by increases in horizontal locomotion or grooming activities (Lutz et al., 2010; Gralewicz and Wiaderna, 2001; Gralewicz et al., 1997b). Statistically significant increases in horizontal locomotion were observed in short-term studies assessing open field behavior following inhalation exposure to 1,2,4-TMB or 1,3,5-TMB (Lutz et al., 2010; Gralewicz and Wiaderna, 2001). Non-monotonic increases in grooming were reported following short-term exposure to 1,2,4-TMB, with

the largest effects observed at 492 mg/m$^3$ and a lack of statistically significant changes at 1,230 mg/m$^3$, although changes in horizontal locomotion were not statistically significant (increases of 3–35% were also non-monotonic) (Gralewicz et al., 1997b). No statistically significant effects on open field activity have been observed following short-term exposure of male rats to 1,2,3-TMB (Lutz et al., 2010; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998). No short-term inhalation exposure studies evaluated open field behaviors immediately after exposure. Open field locomotion following injections with the stimulant amphetamine was amplified by previous short-term exposure to 1,2,3-TMB, but not 1,2,4-TMB (which actually tended to inhibit amphetamine-induced increases in activity at 492 mg/m$^3$), suggesting possible effects of 1,2,3-TMB on sensitization-type responses. As open field testing was conducted 14 or 25 days after termination of repeated exposures for 4 weeks in these studies and TMB isomers are cleared rapidly from the body following the end of inhalation exposures (Section C.2, Appendix C), the results suggest persistence of the effects of 1,2,4-TMB and 1,3,5-TMB on anxiety and/or motor function following clearance of the toxic moiety from the nervous system.

Slight, transient increases in locomotor activity were also observed in open field tests immediately following acute, oral exposure to the TMB isomers (Table 1-3). Significant increases in locomotor activity—measured as number of squares crossed after exposure compared with prior to exposure—were observed at 3,850 mg/kg for 1,2,4-TMB and 1,2,3-TMB, and at ≥1,920 mg/kg for 1,3,5-TMB, with minimal concentration-effect or time-effect relationships and negligible differences in the magnitude of the change in activity between isomers (Tomas et al., 1999b). Increases in locomotor activity were biphasic in nature. At early timepoints immediately following acute exposure, increased locomotor activity was associated with perturbed motor coordination and tremor, whereas after 90 minutes, this apparent motor ataxia progressed to hindlimb paralysis, full immobility, and respiratory distress (e.g., tachypnea), leading to several deaths by 24 hours (Tomas et al., 1999b).

As mentioned previously, open field tests cannot easily distinguish between anxiety-related responses and changes in motor activity. However, mixed effects on motor activity were observed following inhalation exposure to elevated concentrations of TMBs in several studies, and some findings appear inconsistent with observations in open field tests. No consistent treatment-related effects on motor activity were reported in male rats exposed to up to 1,500 ppm C9 fraction (approximately 4,059 mg/m$^3$ TMB isomers) for 13 weeks (Douglas et al., 1993). Transient increases in motor activity (horizontal movement and total activity) were observed in rats exposed to 1,500 ppm C9 at minutes 10–20 of the test during week 9 of the exposure period. However, motor activity in this exposure group returned to control levels during minutes 20–30 of the test, and no effects were observed at the termination of exposure (i.e., week 13). When results were summarized across the entire 30-minute test period, no effects on motor activity were reported at any time during the 13-week exposure period. Decreased motor activity was observed in male rats immediately after exposure to 5,000 mg/m$^3$ 1,2,4-TMB or similar levels of the C9 aromatic fraction

(Mckee et al., 2010). Decreased motor activity was also reported in rats acutely exposed via inhalation to a mixture containing TMB isomers (Lammers et al., 2007), but the use of a mixture precludes a determination of the toxicity specifically associated with individual isomers. As biphasic changes in activity (i.e., initial increases followed by decreases in movement) are frequently observed immediately following acute exposures to solvents, it is likely that the timing of the evaluations across studies, as well as the differing isomer concentrations, may influence the consistency of these results.

Overall, exposure to 1,2,4-TMB and 1,3,5-TMB affects anxiety and/or motor function at concentrations above 492 mg/m$^3$, although the exact, sometimes non-monotonic, concentration-response relationship remains unclear, and the potential contribution of motor activity changes based on activity tests of longer duration were mixed and difficult to interpret. The results for 1,2,3-TMB are difficult to interpret, as no effects were observed following short-term inhalation exposure, while acute oral exposure elicited responses consistent with 1,2,4-TMB and 1,3,5-TMB. Although an explanation for this disparity is lacking, these data highlight a potential difference between 1,2,3-TMB and the other isomers, regarding altered motor function and/or anxiety.

***Cognitive function***

Cognitive function following exposure to TMB isomers alone has not been extensively evaluated in humans or following oral exposure in animals. Controlled exposure of volunteers to mixtures containing TMBs did not indicate any effects on short-term learning or memory tests (Lammers et al., 2007). However, in one study, solvent-exposed construction workers were observed to have decreased performance in memory tasks (Tang et al., 2011). In animals, short-term spatial memory (radial maze performance) was unaffected by exposure to either 1,2,4-TMB or 1,3,5-TMB via inhalation (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Gralewicz et al., 1997b). Similarly, although one study indicates a significant decrement in radial maze performance following exposure to 123 mg/m$^3$ 1,2,3-TMB (Wiaderna et al., 1998), higher concentrations had no effect (Wiaderna et al., 1998), preventing interpretations regarding the significance of this finding. In contrast, effects on cognitive function in passive and active avoidance tests of conditioning behaviors were consistently observed across multiple studies in male rats 6–8 weeks following short-term inhalation exposure to the TMB isomers, although clear concentration-effect relationships were not observed (Table 1-2; Figure 1-1). Comparing the results of the behavioral tests reveals that there are differences in cognitive effects reported for each TMB isomer, as well as differences in the concentrations at which the cognitive effects were observed.

In the passive avoidance tests, rats were conditioned to avoid stepping down from a small, elevated platform (the impulse of rats is to step down in order to escape the bright light and constrained, elevated space of the platform) through the use of a brief series of footshocks applied on the lower level. It is important to clarify that these tests are distinct from tests of pain sensitivity and that observations of decreased step-down latency in these tests do not contrast with the increases in paw-lick latency observed in hot plate tests; in fact, they may be complementary (see

below; note: the footshocks used are of a much shorter duration than those used to induce decreased pain sensitivity in the hot plate tests). Decreases in step-down latency in passive avoidance tests, particularly at 7 days following footshock conditioning, were observed 6–7 weeks after short-term inhalation exposure to ≥123 mg/m$^3$ 1,2,3-TMB and 1,3,5-TMB or ≥492 mg/m$^3$ 1,2,4-TMB (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998; Gralewicz et al., 1997b). Differences in latency prior to footshock were not observed. Decreases in latency were consistently observed and were similar in magnitude across all studies at 7 days post-footshock, although the decreases were not statistically significant for 1,2,4-TMB or 1,2,3-TMB in the study by Gralewicz and Wiaderna (2001). At 3 days post-footshock, decreases in latency were less consistent (i.e., statistically significant decreases were observed at 123 mg/m$^3$ 1,2,3-TMB and at 492 mg/m$^3$ 1,2,4-TMB, but not at other concentrations, and were not observed following exposure to 1,3,5-TMB), and only 123 mg/m$^3$ 1,2,3-TMB was shown to have an effect at 1 day after footshock. In these tests, the effects occurring several days following conditioning with footshock are possibly attributable to a reduced ability to inhibit motor reactions (or a lowered motor threshold) in response to the fear-inducing environment. Alternative explanations involve possible contributions of the following in the TMB-exposed rats: diminished fear response to the footshock; decreased pain sensitivity leading to a less-effective negative reinforcement by the (less painful) footshock; or diminished retention of the fear-associated memory (i.e., from the footshock). However, as statistically significant changes were observed ≤24 hours following footshock only after exposure to 123 mg/m$^3$ 1,2,3-TMB, neither diminished fear responses to the footshock nor decreases in pain sensitivity are likely to be the sole driver(s) of these effects. This suggests that, in this particular test paradigm, TMB exposure causes latent effects on neurological functions associated with the persistence of adaptive behaviors to a fear-inducing stimulus. Despite the consistency of the results at 7 days post-footshock, these tests are insufficient to pinpoint whether the effects of TMB exposure are specific to diminished memory retention, increased impulsivity, and/or decreased motor control.

Reduced performance in two-way active avoidance tests was observed in male rats following short-term inhalation exposure to ≥492 mg/m$^3$ 1,2,4-TMB (Gralewicz and Wiaderna, 2001; Gralewicz et al., 1997b), ≥123 mg/m$^3$ 1,3,5-TMB (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001), and 492 mg/m$^3$ 1,2,3-TMB (Wiaderna et al., 1998). The effects of TMBs were particular to the learning component of the test (acquisition/training session), rather than the memory component (retention session 7 days later) (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998). The conditioning or training of active avoidance behaviors was based on avoiding a painful footshock (the unconditioned stimulus) upon presentation of a tone (conditioned stimulus). Similar to the interpretation of results from passive avoidance tests, it is unclear whether and to what extent potential alterations in locomotor activity (rats had to shuttle between compartments) and/or pain sensitivity following exposure to TMB isomers could contribute to learning deficits in these tests.

Acute inhalation exposure studies provide some support for the observed effects of TMB isomers on learned behaviors. Significant increases in response latency in psychomotor tasks, observed immediately after exposure (effects did not persist to 24 hours later), were reported in male rats following acute exposure to 5,000 mg/m$^3$ 1,2,4-TMB (Mckee et al., 2010) or to 4,800 mg/m$^3$ of a mixture containing TMBs (Lammers et al., 2007). The effects on active and passive avoidance behaviors indicate that learning and/or long-term memory processes are affected by exposure to the TMB isomers. The data suggest that 1,3,5-TMB may be a more potent inducer of toxic effects on cognitive function than 1,2,4-TMB and 1,2,3-TMB, as the effects following exposure to 1,3,5-TMB were more consistent and sometimes occurred at lower concentrations than those reported following exposure to the other two isomers. Overall, however, these differences between isomers were slight.

Controlled human exposure studies suggest that exposures of ≤123 mg/m$^3$ of the TMB isomers do not cause overt CNS depression (measured as heart rate and respiration) (Järnberg et al., 1996), although symptoms related to this effect (e.g., lightheadedness, fatigue) have been reported in workers occupationally exposed to mixtures containing TMBs. In mice, CNS depression has been observed following acute inhalation exposure to >25,000 mg/m$^3$ 1,3,5-TMB, with similar effect levels for 1,2,4-TMB (ACGIH, 2002).

***Other Sensory-related behaviors***

Very little information exists for TMB isomers or mixtures containing TMBs regarding the association between exposure and decrements in sensory-related behaviors (typically visual and/or auditory function). Evidence from a number of occupational studies indicate that exposure to complex solvent mixtures possibly containing TMB isomers can result in visual and auditory dysfunction as measured by the latencies of evoked potentials (Juárez-Pérez et al., 2014; Fuente et al., 2013; da Silva Quevedo et al., 2012; Gong et al., 2003; Pratt et al., 2000). Although acute inhalation studies of rats exposed to TMBs or TMB mixtures observed some effects on visual discrimination tasks (McKee et al., 2010; Lammers et al., 2007), these studies incorporated components of cognition (e.g., learning or habituation; memory) in their design, complicating interpretations of possible effects on sensory function alone. The C9 fraction, a complex mixture of VOCs, including TMB isomers, did not affect the auditory startle response in rats exposed up to 1,500 ppm (approximately 4,059 mg/m$^3$ TMBs) (Douglas et al., 1993). Overall, the data are inadequate to interpret the potential for TMB exposure to affect sensory-related behaviors.

***Electrocortical activity***

The only electrophysiological data available from TMB isomer or mixture studies were measures most likely to be associated with attention, alertness, or memory-related behaviors. Neurophysiological evidence from short-term inhalation studies in animals, as well as supportive evidence from acute oral and injection studies, suggests that exposures to TMB isomers at lower concentrations (at least for 1,2,4-TMB) may affect parameters associated with brain excitability.

Decreases in a particular component of electrocortical arousal (i.e., spike-wave discharge [SWD], bursts in recordings from cortical-hippocampal electroencephalograms [EEGs]) were observed in male rats 120 days after short-term exposure to ≥492 mg/m$^3$ 1,2,4-TMB (statistically significant at 1,230 mg/m$^3$), suggesting persistent functional changes in the rat CNS (Gralewicz et al., 1997a). Altered EEG patterns can be induced by anesthetics as well as stimuli that produce arousal, and may precede other measures of neurotoxicity (U.S. EPA, 1998). In recordings from rats that were awake, but immobile (not exhibiting pronounced exploratory activity, as determined by EEG morphology), statistically significant decreases in the frequency of SWD episodes were observed at 24 hours following short-term exposure to 492 mg/m$^3$ 1,2,4-TMB (decreases that were not statistically significant were also observed at ≥492 mg/m$^3$ 1,2,4-TMB at 30 and 120 days after exposure) (Gralewicz et al., 1997a).

Complementing these findings, dose-related decreases in the duration and number of SWD bursts (termed high-voltage spindles) were observed in rats at ≥240 mg/kg of the TMB isomers subsequent to acute oral exposure (Tomas et al., 1999a) (Table 1-3). The stronger and more persistent effects on electrocortical activity followed a pattern of 1,2,3-TMB > 1,3,5-TMB > 1,2,4-TMB (Tomas et al., 1999a). Similarly, electrophysiological alterations in cortical and hippocampal EEGs were more pronounced following intraperitoneal (i.p.) injection of 1,2,3-TMB, with 1,2,4-TMB and 1,3,5-TMB exerting lesser effects (Tomas et al., 1999c). Although it is unclear whether these changes affect related processes such as memory and seizure initiation/propagation, the observed EEG abnormalities following inhalation (Gralewicz et al., 1997a), oral (Tomas et al., 1999a), and i.p. (Tomas et al., 1999c) exposure to TMB isomers provide supportive evidence of possible acute CNS depression by TMB isomers (Tomas et al., 1999a; Tomas et al., 1999c) and indicate persistent (up to 120 days post-exposure) (Gralewicz et al., 1997a) alterations in CNS activity.

**Table 1-2. Evidence pertaining to neurological effects of TMBs in animals—inhalation exposures**

| Study design[a,b] and reference | Assay and results (as response relative to control) |
|---|---|
| ***1,2,4-TMB*** | |
| ***Pain sensitivity*** | |
| 0, 123, 492, 1,230 mg/m$^3$ (recovery: 1,230 mg/m$^3$ at 2 wks post-exposure)<br>90 d; Rat, Wistar, male, N = 10<br>Korsak and Rydzyński (1996),<br>Table C-28[c] | Hot plate (exposure-dependent increase in paw-lick latency, which recovers by 2 wks post-exposure)<br>*Response immediately post-exposure:* 0, 18, 79*, 95*%<br>*Response at 2 wks post-exposure:* 0, ND, ND, 12% |
| 0, 492 mg/m$^3$<br>4 wks; Rat, Wistar, male, N = 11<br>Gralewicz and Wiaderna (2001),<br>Table C-24 | Hot plate (increased paw-lick latency 24 hrs after footshock)<br>*Response at 50 d post-exposure:* 0, 206%<br>*Response at 50 d post-exposure seconds after footshock:* 0, 25%<br>*Response at 51 d post-exposure 24 hrs after footshock:* 0, 191*% |

| Study design[a,b] and reference | Assay and results (as response relative to control) |
|---|---|
| 0, 123, 492, 1,230 mg/m$^3$<br>4 wks; Rat, Wistar, male, N = 15<br>Gralewicz et al. (1997b), Table C-22 | Hot plate (increased paw-lick latency 24 hrs after footshock[d])<br>*Response at 50 d post-exposure:* 0, −6, 7, −9%<br>*Response at 50 d post-exposure seconds after footshock:* 0, −8, 17, −11%<br>*Response at 51 d post-exposure 24 hrs after footshock:* 0, 2, 74*, 33*% |
| ***Neuromuscular function and coordination*** | |
| 0, 123, 492, 1,230 mg/m$^3$ (recovery: 1,230 mg/m$^3$ at 2 wks post-exposure)<br>90 d; Rat, Wistar, male, N = 10<br>Korsak and Rydzyński (1996), Table C-28 | Rotarod (exposure-dependent increase in percent failures at 13 wks, which does not recover by 2 wks post-exposure)<br>*Response after 13 wks of exposure:* 0, 10, 20, 40*%<br>*Response at 2 wks post-exposure:* 0, ND, ND, 30% |
| ***Motor function and/or anxiety*** | |
| 0, 123, 492, 1,230 mg/m$^3$<br>4 wks; Rat, Wistar, male, N = 15<br>Lutz et al. (2010), Table C-33 | Open field (increased horizontal locomotion (distance traveled); no overall effects with amphetamine challenge[e])<br>*Response at 2 wks post-exposure with no challenge:* 0, 100, 84, 154*%<br>*Response to single amphetamine injection challenge:* 0, 90, −25, 69%<br>*Response to challenge after conditioning:* 0, 43, −50, 31% |
| 0, 492 mg/m$^3$<br>4 wks; Rat, Wistar, male, N = 11<br>Gralewicz and Wiaderna (2001), Table C-24 | Open field (increased horizontal locomotion; number of crossings)<br>*Response at 25 d post-exposure:* 0, 61*%<br>*No change in exploration (rearings) or grooming episodes* |
| 0, 123, 492, 1,230 mg/m$^3$<br>4 wks; Rat, Wistar, male, N = 15<br>Gralewicz et al. (1997b), Table C-22 | Open field (increased grooming at middle concentration):<br>*Response at 25 d post-exposure:* 0, 82, 147*, 76%<br>*No change in horizontal locomotion (number of crossings) or exploration* |
| ***Cognitive function*** | |
| 0, 492 mg/m$^3$<br>4 wks; Rat, Wistar, male, N = 1<br>Gralewicz and Wiaderna (2001), Table C-24 | Passive avoidance (decreased step-down latency 7 d post-footshock[f])<br>*Response at 39 d post-exposure prior to footshock:* 0, 34%<br>*Response at 42 d post-exposure 1 d after footshock:* 0, −23%<br>*Response at 44 d post-exposure 3 d after footshock:* 0, −51 %<br>*Response at 48 d post-exposure 7 d after footshock:* 0, −43%<br>Note: statistical significance 7 d after footshock was noted after the highest and lowest responder from each group was excluded<br><br>Active avoidance (decreased performance during training; learning)<br>*Trials to reach avoidance criteria at 54–60 d post-exposure:* 0, 58*%<br>*No differences were noted during retraining (retention)*<br><br>Radial maze<br>*No notable change in performance 14–18 d post-exposure* |

| Study design[a,b] and reference | Assay and results (as response relative to control) |
|---|---|
| 0, 123, 492, or 1,230 mg/m$^3$<br>4 wks; Rat, Wistar, male, N = 15<br>Gralewicz et al. (1997b), Table C-22 | Passive avoidance (decreased step-down latency 3–7 d post-footshock)<br>*Response at 39 d post-exposure prior to footshock: 0, 26, 41, −31%*<br>*Response at 42 d post-exposure 1 d after footshock: 0, 95, −28, −87%*<br>*Response at 44 d post-exposure 3 d after footshock: 0, 7, −67*, −36%*<br>*Response at 48 d post-exposure 7 d after footshock: 0, −20, −79*, −47*%*<br><br>Active avoidance (decreased performance during acquisition; learning)[g]<br>*Slower increases in avoidance performance across trials: p < 0.003*<br>*Non-significant decrease in total avoidance responses: p = 0.08*<br><br>Radial maze<br>*No notable change in performance 14–18 d post-exposure* |
| ***Electrocortical activity*** | |
| 0, 123, 492, 1,230 mg/m$^3$<br>4 wks; Rat, Wistar, male, N = 9<br>Gralewicz et al. (1997a), Table C-23 | EEG recordings[h] (decreased SWD bursts/hr)<br>*Response at 120 d post-exposure:* 0, 13, −35, −55*%<br>*No change in global arousal level or in SWD/hr at 1 or 30 d post-exposure* |
| ***1,2,3-TMB*** | |
| ***Pain sensitivity*** | |
| 0, 123, 492, 1,230 mg/m$^3$ (recovery: 1,230 mg/m$^3$ at 2 wks post-exposure)<br>90 d; Rat, Wistar, male, N = 10<br>Korsak and Rydzyński (1996), Table C-28 | Hot plate (exposure-dependent increase in paw-lick latency, which recovers by 2 wks post-exposure)<br>*Response immediately post-exposure:* 0, 22*, 68, 78*%<br>*Response at 2 wks post-exposure:* 0, ND, ND, 13% |
| 0, 492 mg/m$^3$<br>4 wks; Rat, Wistar, male, N = 11<br>Gralewicz and Wiaderna (2001), Table C-24 | Hot plate (no statistically significant change in paw-lick latency)<br>*Response at 50 d post-exposure:* 0, 95%<br>*Response at 50 d post-exposure seconds after footshock:* 0, −1%<br>*Response at 51 d post-exposure 24 hrs after footshock:* 0, 75% |
| 0, 123, 492, 1,230 mg/m$^3$<br>4 wks; Rat, Wistar, male, N = 15<br>Wiaderna et al. (1998), Table C-42 | Hot plate (increased paw-lick latency 24 hrs after footshock at middle concentration)<br>*Response at 50 d post-exposure:* 0, −28, −13, −12%<br>*Response at 50 d post-exposure seconds after footshock:* 0, −9, −16, −5%<br>*Response at 51 d post-exposure 24 hrs after footshock:* 0, −19, 45*, 8% |
| ***Neuromuscular function and coordination*** | |
| 0, 123, 492, 1,230 mg/m$^3$ (recovery: 1,230 mg/m$^3$ at 2 wks post-exposure)<br>90 d; Rat, Wistar, male, N = 10<br>Korsak and Rydzyński (1996), Table C-28 | Rotarod (exposure-dependent increase in percent failures at 13 wks, which does not recover by 2 wks post-exposure)<br>*Response after 13 wks of exposure:* 0, 20, 40*, 70*%<br>*Response at 2 wks post-exposure:* 0, ND, ND, 50*% |
| ***Motor function and/or anxiety*** | |
| 0, 123, 492, 1,230 mg/m$^3$<br>4 wks; Rat, Wistar, male, N = 15<br>Lutz et al. (2010), Table C-33 | Open field (statistically significant increase in horizontal locomotion [distance traveled] only after amphetamine challenge[e])<br>*Response at 2 wks post-exposure with no challenge:* 0, 96, 85, 115%<br>*Response to single amphetamine injection challenge:* 0, 15, 198*, 111%<br>*Response to challenge after conditioning:* 0, −21, 103*, 41% |

| Study design[a,b] and reference | Assay and results (as response relative to control) |
|---|---|
| 0, 492 mg/m$^3$<br>4 wks; Rat, Wistar, male, N = 11<br>Gralewicz and Wiaderna (2001), Table C-24 | Open field (no change in horizontal locomotion; crossings)<br>*Response at 25 d post-exposure:* 0, −9%<br>*No change in exploration (rearings) or grooming* |
| 0, 123, 492, 1,230 mg/m$^3$<br>4 wks; Rat, Wistar, male, N = 15<br>Wiaderna et al. (1998), Table C-42 | Open field (no significant change in horizontal locomotion; crossings)<br>*Response at 25 d post-exposure*: 0, 19, 51, 37%<br>*No statistically significant change[i] in exploration (rearings) or grooming* |
| ***Cognitive function*** | |
| 0, 492 mg/m$^3$<br>4 wks; Rat, Wistar, male, N = 11<br>Gralewicz and Wiaderna (2001), Table C-24 | Active avoidance (decreased performance during training; learning)<br>*Trials to reach avoidance criteria at 54–60 d post-exposure:* 0, 53*%<br>*No differences were noted during retraining (retention)*<br><br>Passive avoidance (no significant change in step-down latency[f])<br>*Response at 39 d post-exposure prior to footshock:* 0, −39%<br>*Response at 42 d post-exposure 1 d after footshock:* 0, −40%<br>*Response at 44 d post-exposure 3 d after footshock:* 0, −23 %<br>*Response at 48 d post-exposure 7 d after footshock:* 0, −28%<br><br>Radial maze<br>*No notable change in performance 14–18 d post-exposure* |
| 0, 123, 492, 1,230 mg/m$^3$<br>4 wks; Rat, Wistar, male, N = 15<br>Wiaderna et al. (1998), Table C-42 | Passive avoidance (decreased step-down latency after footshock)<br>*Response at 39 d post-exposure prior to footshock:* 0, −41, −37, 19%<br>*Response at 42 d post-exposure 1 d after footshock:* 0, −74*, −52, −43%<br>*Response at 44 d post-exposure 3 d after footshock:* 0, −54*, −49, −14%<br>*Response at 48 d post-exposure 7 d after footshock:* 0, −50*, −62*, −37%<br><br>Active avoidance (decreased performance during training; learning)<br>*Trials to reach avoidance criteria at 54–60 d post-exposure:* 0, 3, 41*, 14%<br>*No statistically significant differences noted during retraining (retention)*<br><br>Radial maze (decreased performance at low concentration[j])<br>*Increased errors on trial day 3*: 0, 32*, −28, −4% *& day 5:* 0, 30*, −16, 1%<br>*No notable change in trial duration at any day (14–18 d post-exposure)* |

| Study design[a,b] and reference | Assay and results (as response relative to control) |
|---|---|
| ***1,3,5-TMB*** | |
| ***Pain sensitivity*** | |
| 0, 492 mg/m$^3$<br>4 wks; Rat, Wistar, male, N = 11<br>Gralewicz and Wiaderna (2001),<br>Table C-24 | Hot plate (increased paw-lick latency 24 hrs after footshock)<br>*Response at 50 d post-exposure:* 0, 215%<br>*Response at 50 d post-exposure seconds after footshock:* 0, 26%<br>*Response at 51 d post-exposure 24 hrs after footshock:* 0, 246*% |
| 0, 123, 492, 1,230 mg/m$^3$<br>4 wks; Rat, Wistar, male, N = 12<br>Wiaderna et al. (2002), Table C-43 | Hot plate (increased paw-lick latency 24 hrs after footshock at middle concentration)<br>*Response at 50 d post-exposure:* 0, −6, 36, 24%<br>*Response at 50 d post-exposure seconds after footshock:* 0, −14, 8, −4%<br>*Response at 51 d post-exposure 24 hrs after footshock:* 0, −4, 68*, 18% |
| ***Motor function and/or anxiety*** | |
| 0, 492 mg/m$^3$<br>4 wks; Rat, Wistar, male, N = 11<br>Gralewicz and Wiaderna (2001),<br>Table C-24 | Open field (increased horizontal locomotion; number of crossings)<br>*Response at 25 d post-exposure:* 0, 65*%<br>*No change in exploration (rearings) or grooming* |
| ***Cognitive function*** | |
| 0, 123, 492, 1,230 mg/m$^3$<br>4 wks; Rat, Wistar, male, N = 12<br>Wiaderna et al. (2002), Table C-43 | Passive avoidance (decreased step-down latency 7 d post-footshock)<br>*Response at 39 d post-exposure prior to footshock:* 0, −5, 146, 40%<br>*Response at 42 d post-exposure 1 d after footshock:* 0, 99, 108, 113%<br>*Response at 44 d post-exposure 3 d after footshock:* 0, −32, −41, −40%<br>*Response at 48 d post-exposure 7 d after footshock:* 0, −47*, −53*, −3*%<br><br>Active avoidance (decreased performance during training; learning)<br>*Trials to reach avoidance criteria at 54–60 d post-exposure:* 0, 40*, 35*, 50*%<br><br>Radial maze<br>*No notable change in performance 14–18 d post-exposure* |
| 0, 492 mg/m$^3$<br>4 wks; Rat, Wistar, male, N = 11<br>Gralewicz and Wiaderna (2001),<br>Table C-24 | Passive avoidance (decreased step-down latency 7 d post-footshock[g])<br>*Response at 39 d post-exposure prior to footshock:* 0, −3%<br>*Response at 42 d post-exposure 1 d after footshock:* 0, −61%<br>*Response at 44 d post-exposure 3 d after footshock:* 0, −65%<br>*Response at 48 d post-exposure 7 d after footshock:* 0, −57*%<br>Note: statistical significance 3 d after footshock was noted after the highest and lowest responder from each group was excluded<br><br>Active avoidance (decreased performance during training; learning):<br>*Trials to reach avoidance criteria at 54–60 d post-exposure:* 0, 65*%<br><br>Radial maze<br>*No notable change in performance 14–18 d post-exposure* |

| Study design[a,b] and reference | Assay and results (as response relative to control) |
|---|---|
| ***C9 fraction*** | |
| ***Pain sensitivity*** | |
| 0, 100, 500, 1,500 ppm C9 fraction (approximately 0, 270, 1,353, 4,059 mg/m$^3$ TMB isomers)<br>13 wks; Rat, Wistar, male, N = 18–20<br>Douglas et al. (1993), Table C-21 | Decreased pain sensitivity (increase in paw-lick latency)<br>*Response relative to control*<br>*Exposure period:*<br>*0 wks:* 0, 52**, 34**, 19%<br>*5 wks:* 0, 31, −5, 47%<br>*9 wks:* 0, 0, −4, 9%<br>*13 wks:* 0, 4, −1, 17% |

*, **Significantly different from controls ($p < 0.05$, $p \leq 0.01$).

[a]Rotarod and hot plate tests were administered immediately after termination of exposure or following a 2-week recovery period by Korsak and Rydzyński (1996). EEG recordings were acquired prior to exposure and 1, 30, or 120 days after exposure by Gralewicz et al. (1997a). Motor behavior in an open field (tested for 30 minutes) was assessed 14 days after exposure and re-tested following single and multiple (to induce sensitization) injections with amphetamine for 120 minutes by Lutz et al. (2010). For the remaining studies (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998; Gralewicz et al., 1997b), radial maze tests were administered prior to exposure and on days 14–18 after exposure; open field activity (tested for 5–10 minutes) was assessed prior to exposure and on day 25 after exposure; passive avoidance was tested on days 35–48 after exposure; hot plate sensitivity was assessed on days 50 and 51 after exposure; and active avoidance tests were administered on or after day 54 post-exposure.

[b]In instances where authors reported exposures in ppm, EPA converted these values to mg/m$^3$. See Table P-1 for conversion factor, and individual study summary tables for ppm values. N refers to number animals/group.

[c]Tables referenced in the *Study design and reference* column correspond to study summary tables in Appendix C.

[d]Observations of hot plate latency were made prior to (L1); immediately following (L2); or 24 hours after footshock (L3). Values for L3 in Gralewicz et al. (1997b) were determined from reported values for L1 and the ratio of L3/L1 × 100.

[e]No challenge = prior to amphetamine challenge, evaluated for 30 minutes, and reported as Block 1: statistical significance indicated in study text only; amphetamine challenge-induced activity was measured following a single injection or following a single injection challenge after conditioning with five daily injections and evaluated for 120 minutes.

[f]Results of passive avoidance tests in Gralewicz and Wiaderna (2001) may reflect adjusted data where, due to large individual differences, two rats (the highest and lowest responders to footshock) in each group were excluded. As a result, the exact magnitude of change is assumed to be somewhat inaccurate and statistical comparisons of the modified groups are provided in the above evidence table only as notes.

[g]At 54 days post-exposure, TMB-exposed rats were slower to increase the percentage of avoidance responses across blocks (one block = five trials). This reduction in avoidance responses across blocks appeared to be lowest (although not statistically significant) at 1,230 mg/m$^3$. Rats were also observed to have a lower ($p = 0.08$) number of avoidance responses in the whole 30-trial session.

[h]EEGs were recorded at electrodes implanted in the fronto-parietal cortex and the dorsal hippocampus (one recording from each region was analyzed for each rat).

[i]Dose-dependent increases in exploration and nonlinear increases in grooming were not statistically significant.

[j]Data represent percent change relative to control in same trial day, but statistical significance determined by the authors is based on comparison to trial day 1 responses within the same group.

ND = not determined

Note: For studies other than Korsak and Rydzyński (1996), percent change from control was calculated from digitized data using Grab It! XP software.

**Table 1-3. Evidence pertaining to neurological effects of TMBs in animals—oral exposures**

| Study design[a,b] and reference | Assay and results | | | |
|---|---|---|---|---|
| ***1,2,4-TMB*** | | | | |
| ***Motor function and/or anxiety*** | | | | |
| 0, 960, 1,920, 3,850 mg/kg single gavage<br>Rat, Wag/Rij, male, N = 10<br>Tomas et al. (1999b), Table C-40[c] | Open field (transient increases in locomotor activity):<br>*Response at 20 min after exposure relative to pre-injection controls:*<br>0, 34.1, 57.8, 60.6*%<br>*No significant changes were reported at 10, 30, 40, 50, 60, or 70 min* | | | |
| ***Electrocortical activity*** | | | | |
| 0, 240, 960, 3,850 mg/kg, single gavage<br>Rat, Wag/Rij, male, N = 6<br>Tomas et al. (1999a), Table C-39 | EEG recordings[d] (reduction of the duration and number of high voltage spindle episodes) (response relative to vehicle control): | | | |
| | | 20 min | 40 min | 60 min |
| | *Duration* | 0, −72, −58, −83% | 0, −80*, −97*, −45% | 0, 11, −67, −45% |
| | *Number* | 0, −26, −44, −62*% | 0, −53*, −88*, −73*% | 0, 7, −53*, −22% |
| ***1,2,3-TMB*** | | | | |
| ***Motor function and/or anxiety*** | | | | |
| 0, 960, 1,920, 3,850 mg/kg single gavage<br>Rat, Wag/Rij, male, N = 10<br>Tomas et al. (1999b), Table C-40 | Open field (transient increases in locomotor activity):<br>*Response at 20 or 30 min after exposure relative to pre-injection controls:* 0, 30.9, 26.5, 56.1*% (increased 65.6*% at 30 min in at the highest concentration<br>*No significant changes were noted at 10, 40, 50, 60, or 70 min* | | | |
| ***Electrocortical activity*** | | | | |
| 0, 960, 3,850 mg/kg, single gavage<br>Rat, Wag/Rij, male, N = 6<br>Tomas et al. (1999a), Table C-39 | EEG recordings[d] (reduction of the duration and number of high voltage spindle episodes) (response relative to vehicle control): | | | |
| | | 20 min | 40 min | 60 min |
| | *Duration* | 0, −86, −97*, −76*% | 0, −95, −98*, −97*% | 0, −81, −94*, −99*% |
| | *Number* | 0, −71*, −86*, −48% | 0, −84*, −93*, −86*% | 0, −70*, −99*, −96*% |
| ***1,3,5-TMB*** | | | | |
| ***Motor function and/or anxiety*** | | | | |
| 0, 960, 1,920, 3,850 mg/kg single gavage<br>Rat, Wag/Rij, male, N = 10<br>Tomas et al. (1999b), Table C-40 | Open field (transient increases in locomotor activity):<br>*Response at 20 min after exposure relative to pre-injection controls:*<br>0, 0, 46.7*, 42.4*% (increased 65−70% at 40−60 min at the highest concentration<br>*No significant changes were noted at 10, 30, or 70 min* | | | |

| Study design[a,b] and reference | Assay and results | | | |
|---|---|---|---|---|
| ***Electrocortical activity*** | | | | |
| 0, 240, 960, 3,850 mg/kg, single gavage<br>Rat, Wag/Rij, male, N = 6<br>Tomas et al. (1999a), Table C-39 | EEG recordings[d] (reduction of the duration and number of high voltage spindle episodes) (response relative to vehicle control): | | | |
| | | 20 min | 40 min | 60 min |
| | *Duration* | 0, −76*, −79, −86% | 0, −85*, −97*, −95*% | 0, −66*, −94*, −88*% |
| | *Number* | 0, −57, −67, −77% | 0, −52*, −93*, −91*% | 0, −49*, −91*, −89*% |

*Significantly different from controls ($p < 0.05$).

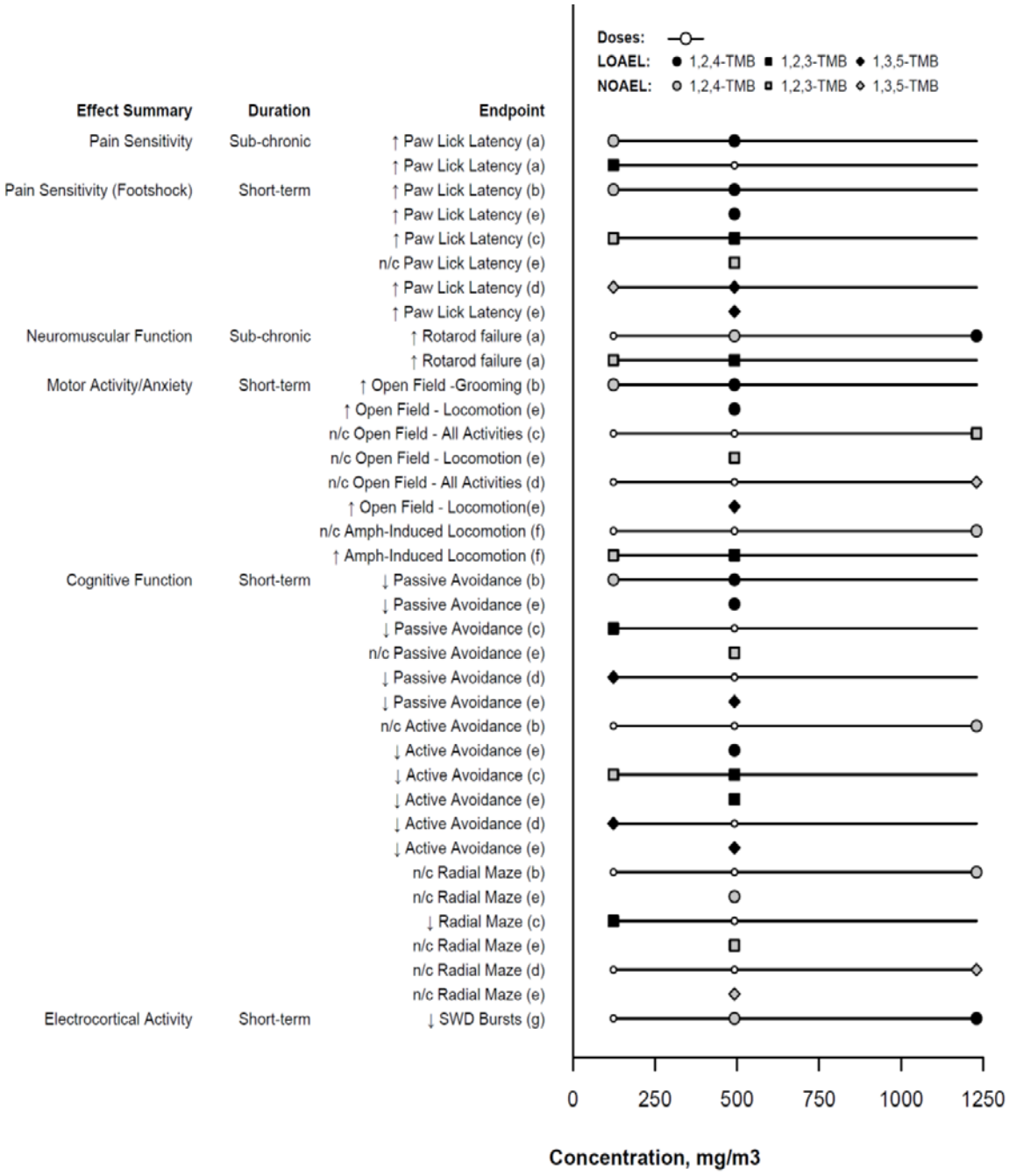
[a]Locomotor activity in open field tests and electrocortical arousal were assessed prior to exposure and immediately after exposure every 10 minutes for up to 70 minutes.

[b]In instances where authors reported exposures in ppm, EPA converted these values to mg/m$^3$. See Table P-1 for conversion factor, and individual study summary tables for ppm values. N refers to number of animals/group.

[c]Tables referenced in the *Study design and reference* column correspond to study summary tables in Appendix C.

[d]EEGs were recorded prior to exposure and at 20, 40, and 60 minutes after exposure via electrodes implanted in the fronto-parietal cortex.

Note: Percent change from control was calculated from digitized data using Grab It! XP software.




Note: Solid lines represent range of exposure concentrations. (a) Korsak and Rydzyński (1996); (b) Gralewicz et al. (1997a); (c) Wiaderna et al. (1998); (d) Wiaderna et al. (2002); (e) Gralewicz and Wiaderna (2001); (f) Lutz et al. (2010); (g) Gralewicz et al. (1997b). All effects are in male Wistar rats. See Table 1-2 for detailed information on when measurements were taken relative to exposure.

**Figure 1-1. Exposure response array of neurological effects following inhalation exposure to 1,2,4-TMB, 1,2,3-TMB, or 1,3,5-TMB.**

***Mode-of-Action Analysis—Neurological Effects***

The observation of neurotoxicity following acute-, short-term-, and subchronic-duration exposure to TMB (Lutz et al., 2010; Lammers et al., 2007; Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998; Gralewicz et al., 1997b; Gralewicz et al., 1997a; Korsak and Rydzyński, 1996; Korsak et al., 1995) may indicate that TMB perturbs normal neurotransmission in exposed animals, although the specific key events necessary for TMB-induced neurotoxicity are not established. Although mechanistic and mode-of-action data are lacking for TMBs, structurally similar compounds like toluene and xylene have been more thoroughly characterized and it is reasonably anticipated that TMBs may interact with similar biological receptors. However, while TMB isomers may operate through similar mechanisms as other alkylbenzenes, the specific neurotoxicological effects mediated via those interactions may be highly disparate. Gralewicz and Wiaderna (2001), citing Kyrklund (1992), note that alkylbenzenes are known to target catecholaminergic systems. Inhalation exposures to toluene and xylene have been shown to significantly change concentration and turnover rate of both dopamine and norepinephrine in various regions of the rat brain (Rea et al., 1984; Andersson et al., 1983; Andersson et al., 1981; Andersson et al., 1980). Although these changes have been hypothesized to be due to the action of potential metabolites on catecholamine receptors (Andersson et al., 1983; Andersson et al., 1981; Andersson et al., 1980), the role of the parent compound has not been fully characterized. Toluene exposure also results in persistent dopamine dysfunction in basal ganglia in chronically exposed rats (Tormoehlen et al., 2014) and styrene can cause decreased dopamine levels and corresponding increases in dopamine receptor expression (Costa, 1996).

The observation of catecholaminergic changes following toluene exposure, and the observation of similar neurological effects following exposure to toluene and TMB isomers, increases the plausibility that the mechanisms of neurotoxicity are similar between the two compounds. For example, subchronic inhalation exposures of rats to low concentrations of toluene (as low as 80 ppm [300 mg/m$^3$]) have been shown to decrease spatial learning and memory, increase dopamine-mediated locomotor activity, increase the number of dopamine D2 receptors, and increase dopamine D2 agonist receptor binding (Hillefors-Berglund et al., 1995; von Euler et al., 1994; von Euler et al., 1993). Dopamine-dependent locomotor hyperactivity has been shown to be biphasic in nature (Riegel and French, 1999), and toluene has also been shown to exert biphasic effects on locomotor activity (Wood and Colotla, 1990; Hinman, 1987). These effects were observed to persist up to 4 weeks after the termination of the toluene exposure. Both of these observations are consistent with the open field behavioral effects observed to persist weeks after short term exposure to 1,2,4- or 1,3,5-TMB, and they are not easily attributed to brain concentrations of the solvents.

Activation of the dopaminergic system may also result in an inability to inhibit locomotor responses (Jackson and Westlind-Danielsson, 1994). Direct application of dopamine to the nucleus accumbens of rats has been observed to result in retardation of the acquisition of passive avoidance

learning at concentrations that also stimulated locomotor activity (Bracs et al., 1984). Increases in catecholaminergic neurotransmission (through exposure to norepinephrine or dopamine agonists) result in dose-dependent reductions in the duration of SWDs in rats (Snead, 1995; Warter et al., 1988). These observations and findings are in concordance with those resulting from exposure to TMBs, including increased locomotion in open field tests and altered SWDs after TMB isomer exposure (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Tomas et al., 1999a; Tomas et al., 1999c; Gralewicz et al., 1997b; Gralewicz et al., 1997a). Effects on the dopamine system, with its well-established role in reinforcement, would also be consistent with the observations of sustained perturbations in active and passive avoidance tasks following short-term TMB exposure. Additionally, with regard to toluene and related aromatic hydrocarbons, it is known that there is direct interaction with these compounds on various ion channels (ligand and voltage gated) that are present in the CNS (Bowen et al., 2006; Balster, 1998).

Exposure to alkylbenzene compounds has also been shown to affect other neurotransmitter pathways. Exposure to benzene, xylene, toluene, and ethylbenzene inhibited N-methyl-D-aspartate (NMDA) receptor function in Xenopus oocytes (Tormoehlen et al., 2014). Toluene was additionally observed to exert dose-dependent effects on rat hippocampal neuron NDMA receptor activity: acute exposures decreased NMDA receptor activity, whereas chronic exposures increased activity, indicating increased expression of NMDA receptors (Tormoehlen et al., 2014). Toluene has additionally been shown to increase the function of gamma-aminobutyric acid (GABA) and glycine receptors and decrease the function of nicotinic acetylcholine receptors (Tormoehlen et al., 2014; Manto, 2012; Win-Shwe and Fujimaki, 2010). Win-Shwe and Fujimaki (2010) concluded that the evidence that toluene inhibits excitatory receptors (NMDA and nicotinic acetylcholine) and enhances inhibitory receptors (GABA and glycine) indicates that toluene exerts a range of both inhibitory and stimulatory effects on exposed animals. By extension, TMB isomers can reasonably be assumed to exert the same neuropharmacological effects in exposed animals, possibly explaining the nonlinearities observed in some endpoints (i.e., effects at lower doses but not higher ones). Further, observations that toluene exposure alters NMDA receptor activities in rat hippocampal neurons may explain the observed effects on the visual system in rodents as well as decreased spatial memory and learning (Win-Shwe and Fujimaki, 2010).

There is not enough information to ascertain the specific molecular sites of action, and how the resultant changes correlate to the observed neurotoxicological effects. However, it is widely believed that the interactions with the neuronal receptors in the brain (e.g., ion channels, catecholaminergic systems) may influence these changes. There is suggestive evidence from functional magnetic resonance imagining (fMRI) (Tang et al., 2011) that exposure to complex solvent mixtures results in deficits in the brain neuronal circuitry responsible for attention and working memory tasks. In exposed subjects, performance in memory tasks was significantly decreased relative to controls, and activity in brain regions responsible for attention and working memory (anterior cingulate, prefrontal, and parietal cortices) was also significantly lowered.

Aromatic hydrocarbons may also perturb neurotransmitter activity via direct action on the phospholipids that comprise nerve cell membranes (Andersson et al., 1981). Perturbation of the phospholipids on the cell membrane could indirectly affect the binding of neurotransmitters to the catecholamine or other receptors and potentially lead to alterations in receptor activity or uptake-release mechanisms. Uneven distribution of the solvent or its metabolites within differing regions of the brain, or spatial variations in phospholipid composition of nerve cell membranes, may explain the differential effects seen in regard to catecholamine levels and turnover (Andersson et al., 1981). Based on effect levels with other related solvents (e.g., toluene) (see Balster (1998)), it is hypothesized that with TMBs, there may be an initial interaction with the neuronal receptors (e.g., catecholaminergic systems, ion channels) followed by, at much higher exposures, interaction with the lipid membrane when the available sites on the neuronal receptors are completely occupied.

Additional mechanisms that may play a role in TMB neurotoxicity include production of reactive oxygen species (ROS). Myhre et al. (2000) observed increased respiratory burst in neutrophils after 1,2,4-TMB exposure demonstrated by fluorescence spectroscopy, hydroxylation of 4-hydroxybenzoic acid, and electron paramagnetic resonance spectroscopy. The authors suggested that the observation of solvent-induced ROS production may be relevant to brain injury, as microglia cells and neutrophils have similar respiratory burst characteristics. Stronger evidence of potential ROS-related mechanisms of neurotoxicity was observed in a related study by Myhre and Fonnum (2001) in which rat neural synaptosomes exposed to 1,2,4-TMB produced a dose-dependent increase in ROS and reactive nitrogen species, demonstrated by the formation of the fluorescence of 2'7'-dichlorofluorescein. This observation of ROS production in rat synaptosomes may potentially explain the observed TMB-induced neurotoxicity in acute, short-term, and subchronic inhalation studies. In experiments investigating the neurotoxic effect of toluene exposures, toluene has been shown to increase the concentration of oxygen radicals, with the hippocampus identified as a particularly vulnerable region (Tormoehlen et al., 2014). Increased production of oxygen radicals in the hippocampus of exposed animals possibly explains the observation of decreased performance in memory and learning tasks. Additional evidence for a possible oxidative stress mode of action is the observation that styrene decreases the levels of glutathione in brains of exposed rats (Costa, 1996).

### *Summary of Neurological Effects*

Neurotoxicity is strongly and consistently associated with exposure to TMBs in multiple studies, and these associations are coherent in human populations exposed to mixtures containing TMBs and in laboratory animals exposed to individual TMB isomers. All three TMB isomers are absorbed readily in humans (Järnberg et al., 1998, 1997a; Järnberg et al., 1996), and occupational studies involving exposure to TMBs and other VOCs demonstrate neuropsychological effects (Chen et al., 1999), deficits in short term memory and reduced motor speed/coordination (Lee et al., 2005), abnormal fatigue (Norseth et al., 1991), and nervousness, anxiety, and/or vertigo (Bättig et

al. (1956), as reviewed by MOE (2006) and Bättig et al. (1958)). These effects, however, cannot be attributed to any specific compound. None of the available human studies have addressed the potential for latent neurological effects, and no TMB-specific studies examined the potential for neurological effects in sensitive populations.

There is strong, consistent evidence of neurotoxicity in male Wistar rats exposed to any TMB isomer via inhalation across multiple concentrations and multiple exposure durations; however, the studies were all conducted at the same institute (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998; Gralewicz et al., 1997b; Gralewicz et al., 1997a; Korsak and Rydzyński, 1996; Korsak et al., 1995). These studies were well-conducted and used common neurobehavioral assays relevant to neurotoxicity in humans. Across studies and TMB isomers, an array of behavioral effects were consistently induced by exposure which, when taken together, provide internally consistent evidence of neurotoxicity amongst the disparate adverse effects. Many of these changes exhibited a large response magnitude (e.g., 150–200% change compared to controls) and, although infrequently tested, increased in magnitude with increasing exposure duration (i.e., tests of pain sensitivity and neuromuscular function). Some endpoints exhibited clear exposure-response relationships, including measures of pain sensitivity and neuromuscular function, when tested immediately after exposure. Most other endpoints did not show a clear concentration-effect relationship, although the direction and magnitude of responses was relatively consistent across studies. In most cases, effects at 1,230 mg/m$^3$ were less robust than those observed at lower TMB concentrations (i.e., responses were nonlinear). However, nonlinear relationships are not uncommon for solvents and, as they were observed across multiple studies using each of the three isomers, they are considered to be biologically-relevant observations rather than experimental artifacts, and this does not necessarily detract from the evidence. By gavage, similar effects were observed (e.g., altered EEG recordings; increased locomotor activity in open field tests) (Tomas et al., 1999a; Tomas et al., 1999b), although testing by this route was not as extensive as by inhalation. Altogether, these data support a high level of confidence in the conclusion that inhalation exposure to TMB isomers causes neurotoxicity.

Considering all of the available data, there is a strong indication for a lack of reversibility of TMB-induced neurotoxicity. Although findings of reduced pain sensitivity in naïve animals appeared to be mostly reversible 2 weeks after subchronic exposure to 1,2,4-TMB or 1,2,3-TMB (Korsak and Rydzyński, 1996) and no decrease in pain sensitivity was observed in rats exposed to the C9 fraction when tested 24 hours following the termination of exposure (Douglas et al., 1993), a variety of other neurotoxic findings contradict this suggestive evidence of reversibility. Specifically, statistically significant effects on measures of pain sensitivity (i.e., following footshock challenge), neuromuscular function, open field activity, and cognitive function (learning and memory) were all observable several weeks to months after short-term or subchronic exposure to the various TMB isomers. For pain sensitivity in particular, the possibility of reversibility following subchronic exposure was only tested at the highest concentration of TMB used in the Korsak and Rydzyński

(1996) study (i.e., 1,230 mg/m$^3$). In multiple other tests of neurological function (including pain sensitivity following a footshock challenge), it was shown that exposure to any of the TMB isomers can show a non-monotonic, dose-response relationship when tested some period of time after exposure, with 1,230 mg/m$^3$ TMB usually resulting in no response or a substantially reduced response as compared to lower TMB concentrations (e.g., 492 mg/m$^3$). Thus, from the data available, a determination regarding the reversibility of TMB-induced decreases in pain sensitivity at other concentrations (i.e., 492 mg/m$^3$) at 2 weeks post-exposure cannot be made with confidence. Additionally, while decreased pain sensitivity is observed to possibly resolve following termination of exposure, the determination of reversibility is based solely on statistical significance. In both the Korsak and Rydzyński (1996) and Douglas et al. (1993) studies, latency to paw-lick was still increased relative to control after termination of exposure in the high-dose animals: 12–13% 2 weeks after exposure to 1,230 mg/m$^3$ 1,2,4-TMB or 1,2,3-TMB, and 37% 24 hours after exposure to 1,500 ppm C9 fraction (approximately 4,059 mg/m$^3$ TMB isomers). While these effects are not statistically significant, they may still represent biologically significant impairments in pain sensitivity at time points following the termination of exposure.

Other evidence from TMB isomer studies demonstrates that decreased pain sensitivity in particular, and neurotoxicity in general, is not a transient effect of exposure, and that these effects are long-lasting (potentially permanent) in exposed rats. In several short-term studies investigating the neurotoxicity of all of the TMBs individually, a footshock challenge was incorporated into the testing paradigm in order to test whether TMB exposure resulted in long-lasting, latent effects. Footshock itself reduces pain sensitivity and when applied to all groups (exposed animals and controls), it should elicit a similar response. By measuring pain sensitivity 24 hours following the footshock challenge, the short-term studies observed that treated animals retained decreased pain sensitivity, whereas control animals' responses had returned to background levels. This demonstrates that exposure to TMB results in long-lasting latent changes in how the nervous system is able to respond to negative environmental stimuli. It is important to consider that an environmental challenge was not included in the design of Korsak and Rydzyński (1996). If it had been, it can be reasonably assumed that the animals exhibiting reduced pain sensitivity immediately after exposure in Korsak and Rydzyński (1996) would also exhibit the persistent, latent neurotoxicity observed in the short-term studies. Of particular note is that this evidence indicates that effects on nervous system processes associated with pain sensitivity are not rapidly reversible or associated with clearance of the chemical from the body. TMB isomers have been observed to clear rapidly from blood (Section C.2, Appendix C), and decreased pain sensitivity following footshock persisted 51 days after termination of short-term exposures (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Gralewicz et al., 1997b). The observed effects on pain sensitivity 51 days after exposure cannot be related to the presence of TMB isomers in the blood given the TMB isomers' half-lives of elimination from venous blood (4.6–10 hours) (Świercz et al., 2016; Swiercz et al., 2006; Swiercz et al., 2003; Swiercz et al., 2002). Even when considering the

longest half-life of elimination (10 hours for 1,2,4-TMB), >99% of the chemical would be eliminated from blood 70 hours after termination of exposure (i.e., approximately 3 days). As pointed out in *A Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002), "[i]t is also important to keep in mind that effects that may initially appear to be reversible may re-appear later or be predictive of later adverse outcomes." (pg. 4-16). Additionally, the *Guidelines for Neurotoxicity Risk Assessment* (U.S. EPA, 1998) state that "latent effects (those that become evident only after an environmental challenge [e.g., in this case, footshock]) have a high level of concern." These consistent latent effects on pain sensitivity after footshock are considered adverse. Thus, it is concluded that the neurotoxic changes in rats exposed to TMB isomers are persistent.

The hot plate test is a relatively simple assessment that may not be sensitive enough to detect subtle changes (U.S. EPA, 1998), suggesting that the large changes observed immediately after TMB exposure may represent gross effects. It is possible that, at longer durations after exposure, an environmental challenge is necessary for the more subtle perturbations that persist to become manifest at a detectable level. Decrements in pain sensitivity following footshock appear to reflect a lengthening of the numbing effects of footshock following exposure to TMBs weeks earlier; the immediate effects of footshock, which essentially temporarily impairs the nociceptive function of the nervous system, were unchanged by prior TMB exposure. Although the attribution of these findings to decreased pain sensitivity alone may be complicated by possible effects of TMB exposure on cognition, the results suggest that some aspect(s) of the altered pain sensitivity phenotype fail to resolve following termination of exposure. No environmental challenge was applied in the subchronic study by Korsak and Rydzyński (1996); such an experiment may have uncovered similar latent responses. Conversely, the short-term TMB exposure studies testing pain sensitivity failed to analyze effects shortly after exposure, as these evaluations only occurred at ≥50 days post-exposure.

Some differing results are observed in neurotoxicity tests involving exposure to the C9 fraction (Douglas et al., 1993). It is notable, however, that not all of the C9 studies' neurological endpoints failed to show an effect of exposure: exposure of male rats to 5,000 mg/m$^3$ C9 fraction or 1,2,4-TMB resulted in similar decrements in motor activity immediately after exposure (Mckee et al., 2010). As the C9 fraction is comprised of multiple solvents, the effects on motor activity due to 1,2,4-TMB in the mixture are more pronounced than the effects due to exposure to the isomer alone. However, the observed differences in other neurotoxicological tests may be due to differences in the neurotoxicity of single isomers of TMB as compared to a mixture of multiple aromatic compounds including TMB isomers. As the specific modes of action associating any of the various neurotoxic endpoints with exposure to TMB isomers or to the other components of the mixture are not known, it is plausible that there are mechanisms that modify the observed phenotypes with mixtures exposure. Alternatively, it is also possible that differences in experimental designs rather than real differences in toxicity are contributing to any observed differences. The experimental details varied substantially between the C9 fraction and TMB isomer

studies. Additionally, the possibility remains that differences in study findings may also reflect differences in study design, as the C9 fraction studies were not conducted in a manner completely similar to the TMB isomer-specific short-term and subchronic studies. Specifically regarding pain sensitivity, when all studies (TMB isomer and C9 studies) are considered in the proposed time-course of effects, the C9 study by Douglas et al. (1993) is consistent with the observed reversibility (to levels not significantly different from controls) of the robust increase in paw-lick after some time has passed since termination of exposure, a finding also observed in Korsak and Rydzyński (1996) and the numerous short-term studies (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998; Gralewicz et al., 1997b). However, whereas these same short-term studies demonstrate that some latent neurotoxic effects of TMB exposure persist long after exposures have ended, including the observation of decreased pain sensitivity following environmental challenge, the C9 studies did not measure these potentially more subtle manifestations of neurotoxicity. Overall, the available data from C9 fraction studies do not reduce the confidence in the conclusion that exposure to TMB isomers results in neurotoxicity.

The spectrum of observed effects suggests that TMBs affect multiple, possibly overlapping, CNS systems rather than a single brain region or neuronal nuclei (suggested by the solvent activity of the compounds). Almost all tests (including pain sensitivity) involve a contributing component of motor system function. However, apart from the consistent observations of TMB exposure-induced decrements in balance and neuromuscular coordination in rotarod tests, none of the identified short-term or subchronic studies on individual TMB isomers employed protocols capable of distinguishing effects on motor activity alone (e.g., the majority of studies used open field tests 5–10 minutes in duration which are strongly influence by anxiety-related behaviors); thus, it remains to be determined to what extent TMB exposure-induced effects on motor system function might explain the spectrum of observed behavioral changes. Similarly, while latent neurological effects following TMB exposure were consistently observed across studies and isomers, these effects were difficult to characterize as deficits in a single neurological function. For example, latent measures of pain sensitivity following TMB exposure, although consistent, were only statistically significant when the rats were challenged with a footshock on the prior day. The most likely explanation for this observation is that TMB exposure extends the duration of footshock-induced decreases in pain sensitivity, since the immediate response to footshock was similar across groups; yet, it cannot be ruled out that TMB exposure could alter cognitive function, resulting in the observed responses.

Evidence from related compounds (i.e., alkylbenzene derivatives such as toluene, styrene, and xylene) supports the neurotoxicological evidence available for TMB isomers. Multiple review articles identified in the literature describe human populations exposed to toluene, styrene, or hydrocarbon solvent mixtures that suffer neurotoxic effects (e.g., color discrimination, neuropsychological symptoms, decreased reaction times, impaired postural equilibrium, etc.) that are functionally similar to those observed in adults exposed to mixtures containing TMB isomers

(Ritchie et al., 2001; Costa, 1996). Unfortunately, sensory-related endpoints have not been well-studied in animals exposed to TMB isomers, preventing conclusions regarding similarities in these types of responses across related compounds. Somewhat less relevant to chronic, low-level exposures but still useful in qualitative hazard identification, evidence from the reviewed inhalant abuse literature (largely focused on toluene) also includes the observation of numerous neurological effects (some of which are similar to those in the TMB database): cerebellar dysfunction (e.g., ataxia), cranial nerve abnormalities, and impairments in memory, attention, and learning (Tormoehlen et al., 2014; Manto, 2012; Arora et al., 2008; Grandjean and Landrigan, 2006). Evidence from the integrated reviews of the animal literature also identifies effects such as ototoxicity, altered operant conditioning, and increased locomotor activity in adults resulting from exposure to toluene or xylene (Ritchie et al., 2001).

Evidence from related compounds also helps identify possible gaps in the current TMB database, the largest of which is uncertainty surrounding the degree to which TMB isomers can be expected to result in developmental neurotoxicity. Although no studies on the possible developmental neurotoxicity of individual TMB isomers exist, the potential for developmental neurotoxicity has been studied in related compound and/or mixture studies. In a study investigating developmental neurotoxicity due to exposure to a mixture containing TMB isomers (i.e., Aromatol, a mixture of ethyltoluenes and TMBs indicated by the study authors to be a mild CNS depressant), no signs of developmental neurotoxicity (startle reaction time, righting response, open field behavior, or altered avoidance tasks) were observed in rats exposed gestationally to concentrations of up to 2,000 mg/m$^3$ Aromatol (Lehotzky et al., 1985), although methodological details and data were not reported. However, another gestational exposure study of a less informative mixture containing TMBs (i.e., white spirit, which has a lower TMB content and greater proportion of non-TMB compounds) did report long-lasting learning and memory deficits, but not neuromuscular or motor activity changes, at 800 ppm (Hass et al., 1999; Hass et al., 1997; Hass et al., 1995). In addition, suggestive evidence for developmental neurotoxicity does exist when considering other related alkylbenzene compounds. In reviews of the human literature, a range of cognitive, behavioral, and visual dysfunction effects have been observed in children whose mothers were exposed occupationally or via inhalant abuse (Grandjean and Landrigan, 2014; Hannigan and Bowen, 2010; Win-Shwe and Fujimaki, 2010; Grandjean and Landrigan, 2006). Integrated reviews of the animal literature (Hannigan and Bowen, 2010; Bowen and Hannigan, 2006; Ritchie et al., 2001) also found that some developmental neurotoxicity studies (Bowen and Hannigan, 2006; Hass et al., 1999; Hougaard et al., 1999; Hass et al., 1997; Jones and Balster, 1997; Hass et al., 1995) reported that gestational exposures to toluene or xylene resulted in delayed development of the air righting reflex, impaired performance in behavioral tests, sex-specific decrements in spatial learning, or lower absolute brain weights. Notably, the studies that do report effects on developmental neurotoxicity tended to either utilize fairly high exposure concentrations

(500–1,800 ppm) or unconventional dosing paradigms meant to approximate the non-continuous, episodic exposures experienced by inhalant abusers.

In summary, the TMB neurotoxicity database supports a determination that TMBs are neurotoxic following inhalation or oral exposure, based on strong and consistent effects in experimental animals that are coherent with observations in exposed humans; biological plausibility based primarily on similarities to findings from related chemicals; evidence of effects that worsen with increasing duration of exposure; delayed-onset and/or latent neurological effects in animals several weeks following exposure; and observed exposure-response relationships in animals tested immediately after exposure. Further supporting this determination is evidence drawn from the larger alkylbenzene database reporting strong evidence for neurotoxicity in both human and animal studies.

### 1.2.2. Respiratory Effects

There is evidence in humans and animals that inhalation exposure to TMBs induces respiratory toxicity. The human evidence comes from occupational and residential studies involving complex VOC mixtures that include TMBs; thus, effects cannot be attributed to any TMB isomer specifically. TMB isomers are associated with increased measures of respiratory irritation, such as laryngeal and/or pharyngeal irritation (Norseth et al., 1991) and asthmatic bronchitis (Bättig et al. (1956), as reviewed in MOE (2006) and Bättig et al. (1958)) following occupational exposures. Residential exposures have demonstrated significant associations between 1,2,4-TMB and asthma (Billionnet et al., 2011). Controlled human exposures (Jones et al., 2006; Järnberg et al., 1997a; Järnberg et al., 1996) have failed to observe substantial irritative symptoms following acute (<4 hours) inhalation exposures to TMB isomers of up to 25 ppm (123 mg/m$^3$). For full details of the epidemiologic and controlled human exposures studies (including human subjects research ethics procedures), see individual study summary tables in Appendix C.

In animals, there is consistent evidence of respiratory toxicity following inhalation exposure of rodents to the TMB isomers (Table 1-4; Figure 1-2). Markers of inflammation and irritation in the lungs of rats have been observed following subchronic inhalation exposures of Wistar rats to 1,2,4-TMB or 1,2,3-TMB. Increases in immune and inflammatory cells in bronchoalveolar lavage (BAL) fluid have been observed following subchronic exposures of male Wistar rats to 1,2,4-TMB at concentrations ≥123 mg/m$^3$ (Korsak et al., 1997). Specifically, the number of cells in the BAL fluid of exposed rats was increased for both total cells (≥123 mg/m$^3$) and macrophages (≥492 mg/m$^3$). However, some attenuation of these effects was observed at high concentrations (i.e., at 1,230 mg/m$^3$) compared to lower concentrations. For example, the number of macrophages was increased 2.7-fold relative to control at 492 mg/m$^3$, but only 2.2-fold at 1,230 mg/m$^3$. This may indicate either adaptation to the respiratory irritation effects of 1,2,4-TMB, saturation of metabolic pathways, or immune suppression at higher doses. Subchronic exposure of male Wistar rats also significantly increased the BAL fluid content of polymorphonuclear leukocytes and lymphocytes; however, the specific concentrations eliciting these significant increases were not reported by

study authors. A small, but not significant, decrease in cell viability (all cells) was observed following subchronic exposure to 1,2,4-TMB at ≥123 mg/m$^3$ (Korsak et al., 1997).

In addition to increases in immune and inflammatory cells in BAL fluid following exposure to 1,2,4-TMB, histopathological alterations characterized by increases in lymphatic tissue in the lower respiratory tract have also been observed following subchronic exposures of male and female Wistar rats to 1,2,4-TMB or 1,2,3-TMB (Korsak et al., 2000a, b). Significant proliferation of peribronchial lymphatic tissue was observed in male rats exposed to 492 mg/m$^3$ 1,2,4-TMB, although trend analysis demonstrated that this increase was not concentration-dependent due to lack of an effect at the 1,230 mg/m$^3$. Statistically significant increases in interstitial lymphocytic infiltrations were also observed in male rats exposed to 492 mg/m$^3$ 1,2,4-TMB and in male and female rats exposed to 1,230 mg/m$^3$ 1,2,3-TMB or 1,2,4-TMB, respectively. The later increases were concentration-dependent based on trend analysis. Slight increases in pulmonary macrophage infiltration and alveolar wall thickening were also observed in male rats exposed to 450, 900, or 1,800 mg/m$^3$ C9 fraction for 12 months (Clark et al., 1989).

In some 1,2,4-TMB- or 1,2,3-TMB-exposed rats exhibiting peribronchial lymphatic proliferation, the bronchial epithelium lost its cuboidal shape and formed lymphoepithelium (Korsak et al., 2000a, b). However, this formation of lymphoepithelium was apparently non-monotonic and not dependent on concentration. Alveolar macrophages were increased in both sexes exposed to 1,230 mg/m$^3$ 1,2,4-TMB (significant only for males), with trend analysis demonstrating concentration-dependence across the entire concentration range. Goblet cells were statistically significantly increased in a concentration-dependent manner in female rats exposed to ≥492 mg/m$^3$ 1,2,3-TMB. When the incidences of all pulmonary lesions were analyzed in aggregate, lesions were significantly increased in males at 492 mg/m$^3$ 1,2,4-TMB, but not at any concentration in females. However, trend-analysis demonstrated significant increases in aggregate pulmonary lesions in both sexes across the entire concentration range. In rats exposed to 1,2,3-TMB, the aggregate incidences of pulmonary lesions were not statistically significantly increased at any single concentration in males or females. Male rats, however, did exhibit a concentration-dependent increase in aggregate lesions according to trend analysis. Studies on the respiratory effects of subchronic exposures to 1,3,5-TMB were not available.

Additional effects on clinical chemistry including increased total protein (37% increase at exposures of both 123 and 492 mg/m$^3$), decreased mucoprotein (13% decrease at 123 mg/m$^3$ exposure), increased lactate dehydrogenase (170 and 79% increase at 123 and 492 mg/m$^3$, respectively), and increased acid phosphatase activity (47–75% increase at ≥123 mg/m$^3$) were observed in animals exposed to 1,2,4-TMB, suggesting pulmonary irritation or inflammation (Korsak et al., 1997). All of these effects also exhibited either some attenuation of effect at high concentrations compared to lower concentrations. Therefore, some adaptation to the respiratory irritation effects of 1,2,4-TMB may be occurring.

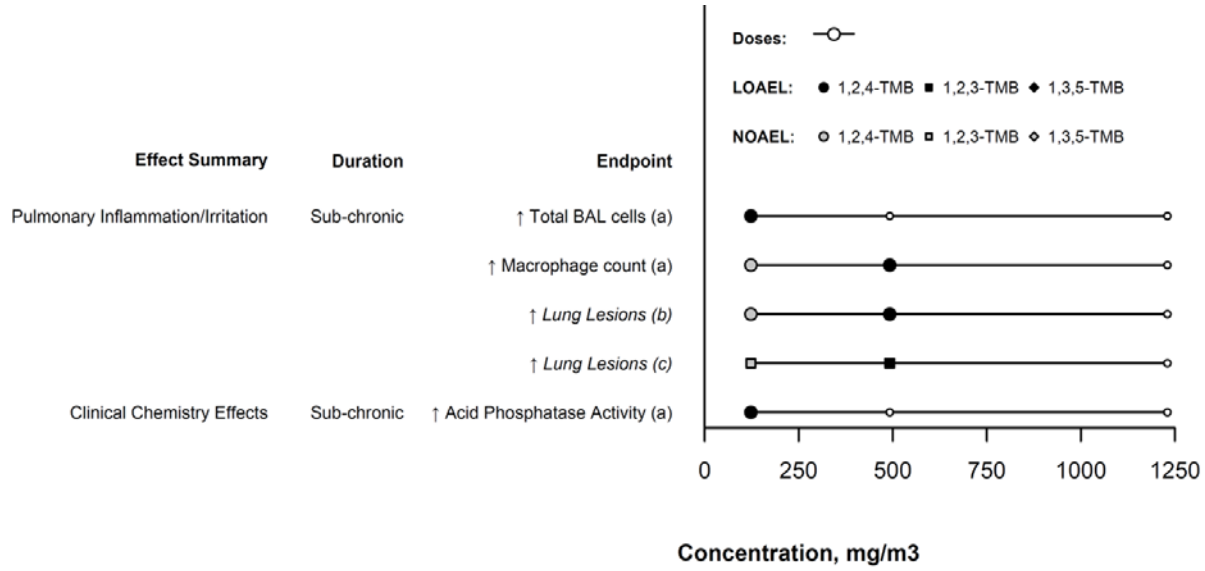
Decreased respiration, a symptom of sensory irritation, has been observed in male BALB/C mice during acute inhalation exposures to individual TMB isomers or Farbasol (a solvent mixture containing TMB isomers) for 6 minutes. These acute exposures were observed to result in dose-dependent depression of respiratory rates, with the maximum decrease in respiration occurring in the first 1 or 2 minutes of exposure (Korsak et al., 1997; Korsak et al., 1995). The concentration of 1,2,3-TMB, 1,2,4-TMB, 1,3,5-TMB, or Farbasol that was observed to result in a 50% depression in the respiratory rate ($RD_{50}$) was similar between the three isomers and the mixture: 578, 541, 519, or 638 ppm (2,844, 2,662, 2,553, or 3,139 mg/m$^3$), respectively.

**Table 1-4. Evidence pertaining to respiratory effects of TMBs in animals—inhalation exposures**

| Study design[a] and reference | Results |
|---|---|
| ***1,2,4-TMB*** | |
| ***Pulmonary inflammation/irritation*** | |
| 0, 123, 492, 1,230 mg/m$^3$, 90 d (6 hrs/d, 5 d/wk)<br>Rat, Wistar, male, N = 6–7<br>Korsak et al. (1997), Table C-29[b] | Increased total bronchoalveolar cell count with evidence of attenuation at high exposure.<br>*Response relative to control:* 0, 202***, 208**, 131*%<br><br>Increased macrophage count with evidence of attenuation at high exposure.<br>*Response relative to control:* 0, 107, 170**, 116**% |
| 0, 123, 492, 1,230 mg/m$^3$, 90 d (6 hrs/d, 5 d/wk)<br>Rat, Wistar, male and female, N = 10<br>Korsak et al. (2000a), Table C-30 | Increase in number of pulmonary lesions.<br>*Response relative to control:* Incidences not reported; thus, calculation of response relative to control not possible; authors report statistically significant increases at 492 and 1,230 mg/m$^3$. |
| ***Clinical chemistry effect*** | |
| 0, 123, 492, 1,230 mg/m$^3$, 90 d (6 hrs/d, 5 d/wk)<br>Rat, Wistar, male, N = 10<br>Korsak et al. (1997), Table C-29 | Increased acid phosphatase activity with evidence of attenuation at high exposure.<br>*Response relative to control:* 0, 47*, 74*, 45*% |
| ***Sensory irritation (decreased respiration)*** | |
| 1,245, 3,178, 5,186, 6,391, 9,486 mg/m$^3$, 6 min<br>Mouse, BALB/C, male, N = 8–10<br>Korsak et al. (1997); Korsak et al. (1995), Tables C-29 and C-27 | Decreased respiratory rate as measured during first minute of exposure.<br>*Response relative to control:* $RD_{50}$ = 2,844 |

| Study design[a] and reference | Results |
|---|---|
| ***1,2,3-TMB*** | |
| ***Pulmonary inflammation/irritation*** | |
| 0, 123, 492, 1,230 mg/m$^3$, 90 d (6 hrs/d, 5 d/wk)<br>Rat, Wistar, male and female, N = 10<br>Korsak et al. (2000b), Table C-31 | Increase in number of pulmonary lesions.<br>*Response relative to control:* Incidences not reported; thus, calculation of response relative to control not possible; authors report statistically significant increases at 492 and 1,230 mg/m$^3$. |
| ***Sensory irritation (decreased respiration)*** | |
| 1,255, 2,514, 4,143, 7,828 mg/m$^3$, 6 min<br>Mouse, BALB/C, male, N = 8−10<br>Korsak et al. (1997), Table C-29 | Decreased respiratory rate as measured during first minute of exposure.<br>*Response relative to control:* $RD_{50}$ = 2,662 |
| ***1,3,5-TMB*** | |
| ***Sensory irritation (decreased respiration)*** | |
| 1,348, 2,160, 2,716, 3,597, 4,900 mg/m$^3$, 6 min<br>Mouse, BALB/C, male, N = 8−10<br>Korsak et al. (1997), Table C-29 | Decreased respiratory rate as measured during first minute of exposure.<br><br>*Response relative to control:* $RD_{50}$ = 2,553 |
| ***C9 fraction*** | |
| ***Pulmonary inflammation/irritation*** | |
| 0, 450, 900, 1,800 C9 fraction (approximately 0, 203, 405, 810 mg/m$^3$ TMB isomers), 12 mo (5 d/wk, 6 hrs/d)<br>Rats, Wistar, male, N = 50<br>Clark et al. (1989), Table C-20 | Increased pulmonary macrophage infiltration<br>*Response relative to control*: 0, 260, 260, 260% (mean severity grade)<br><br>Increased alveolar wall thickening<br>*Response relative to control*: 0, 23, 15, 23% (mean severity grade) |

*Statistically different from controls at $p < 0.05$.
**Statistically different from controls at $p < 0.01$.
***Statistically different from controls at $p < 0.001$.
[a]In instances where authors reported exposures in ppm, EPA converted these values to mg/m$^3$. See Table P-1 for conversion factor, and individual study summary tables for ppm values. N refers to number animals/group.
[b]Tables referenced in Study Design and Reference column correspond to study summary tables in Appendix C.




Note: Solid lines represent range of exposure concentrations. (a) Korsak et al. (1997); (b) Korsak et al. (2000a); (c) Korsak et al. (2000b); (d) Korsak et al. (1995). All effects are in male Wistar rats, except for increased pulmonary lesions, which occur in both male and female Wistar rats. Effects in italics are from studies that reported actual exposure concentrations.

**Figure 1-2. Exposure response array of respiratory effects following inhalation exposure to 1,2,3-TMB, 1,2,4-TMB, or 1,3,5-TMB.**

### *Mode-of-Action Analysis—Respiratory Effects*

Data regarding the potential mode of action for the respiratory effects resulting from TMB inhalation exposures are limited and the key events for TMB-induced respiratory toxicity are not established. However, the available toxicity data suggest that TMB isomers act as potent acute respiratory irritants and induce inflammatory responses following longer exposures (i.e., subchronic) in animals. Korsak et al. (1995) and Korsak et al. (1997) have suggested that decreased respiratory rate following TMB inhalation exposure is indicative of irritation, and proposed that respiratory irritants such as TMB may activate a "sensory irritant receptor" on the trigeminal nerve ending in the nasal mucosa leading to an inflammatory response. Korsak et al. (1997) and Korsak et al. (1995) further suggested that activation of this irritant receptor follows either adsorption of the agonist, or adsorption and chemical reaction with the receptor. The authors referenced a proposed model for the receptor protein that includes two main binding sites for benzene moieties and a thiol group. Further, they suggested that in the case of organic solvents (i.e., toluene, xylene, and TMB), a correlation between the potency of the irritating effect and the number of methyl groups is likely, given the observation that $RD_{50}$ values for depressed respiratory rates following exposure to TMB isomers are approximately 8-fold lower than toluene and 4-fold lower than xylene.

Following subchronic inhalation exposure of rats to 1,2,4-TMB, inflammatory cell (i.e., macrophages, polymorphonuclear leukocytes, and lymphocytes) numbers were increased

along with markers of their activation (i.e., total lactate dehydrogenase and acid phosphatase activity in BAL) (Korsak et al., 1997), further indicating the inflammatory nature of responses in the respiratory tract of TMB-exposed animals. Inflammatory pulmonary lesions were also observed following subchronic inhalation exposures in rats. However, many of these effects were not observed to be concentration-dependent in repeat exposure studies (i.e., no progression of effect over an order of magnitude of concentrations), suggesting that there may be adaptation to respiratory irritation that occurs following extended inhalation exposure to TMB. The processes responsible for the respiratory inflammatory responses observed in subchronically exposed animals are unknown. However, a major inflammatory mediator, interleukin-8 (IL-8), was increased following exposure of porcine and human macrophages to secondary organic aerosol (SOA) particles derived from 1,3,5-TMB (Gaschen et al., 2010). The observation that IL-8 levels increase following exposure to 1,3,5-TMB-derived SOA is noteworthy, as a major function of IL-8 is to recruit immune cells to sites of inflammation. Therefore, the observation of inflammatory lesions involving immune cells (i.e., macrophages and leukocytes) may be partially explained by increases in inflammatory cytokines following TMB exposures. Additionally, ROS generation has been observed in cultured neutrophil granulocytes and rat neural synaptosomes exposed to TMB (Myhre and Fonnum, 2001; Myhre et al., 2000), and the related compounds, benzene and toluene, have been shown to induce oxidative stress in cultured lung cells (Mögel et al., 2011). Although pulmonary ROS generation has not been observed following in vivo or in vitro TMB exposures, there is suggestive evidence that it could play a role in the irritative and inflammatory responses seen in exposed animals.

In a study investigating jet fuel-induced cytotoxicity in human epidermal keratinocytes, aromatic hydrocarbons were more potent inducers of cell death than aliphatic constituents, even though the aromatic compounds only accounted for less than one-fourth of aliphatic constituents (Chou et al., 2003). Of the single aromatic ring hydrocarbons, 1,2,4-TMB and xylene were the most lethal to human epidermal keratinocytes. Increased cytotoxicity may explain the small, but insignificant, decrease in BAL cell viability observed in Korsak et al. (1997).

***Summary of Respiratory Effects***

Respiratory toxicity is associated with inhalation exposure to TMBs based on coherent and consistent evidence in humans and animals. All three TMB isomers are absorbed by humans (Järnberg et al., 1998, 1997a; Järnberg et al., 1996), and occupational and residential studies involving exposure to TMBs and other VOCs suggest an association between TMB exposure and asthmatic symptoms (Billionnet et al., 2011; Bättig et al., 1956) and sensory irritation (Norseth et al., 1991). These effects, however, cannot be attributed to any specific compound, and a causal association cannot be determined. Evidence from related compounds also demonstrates an association between benzene and alkylbenzenes (toluene, xylene, and ethylbenzene) and asthma, increased risk of bronchitis, and decreases in lung function (Bolden et al., 2015).

There is strong, consistent evidence of respiratory toxicity in male and female Wistar rats exposed to any TMB isomer via inhalation across multiple concentrations and multiple durations, although the studies were conducted at the same institute (Korsak et al., 2000a, b; Korsak et al., 1997; Korsak et al., 1995). Some endpoints (i.e., BAL macrophages and alkaline phosphatase [AP]) showed concentration-dependence at low- and mid-exposures; all effects were observed to exhibit some attenuation of effect at high doses, potentially indicating either adaptation to the respiratory irritation effects, saturation of metabolic and/or toxicity pathways, or immune suppression at higher doses. As stated above, the observed respiratory effects following TMB exposure are most likely irritative and/or inflammatory in nature. This conclusion is supported by the nature of the observed lung lesions (increased macrophages, interstitial lymphocytic infiltrations, etc.), but also by the observation of similar hematological effects (increases in white blood cells [WBCs], increased monocytes, etc.). Alterations in red blood cell [RBC] counts may also be attributable to the respiratory irritative and inflammatory effects of TMB isomers. An irritative/inflammatory mode of action for respiratory effects observed at high doses in animal models may increase concern that these effects are relevant to human populations. Although not attributable to individual TMB isomers, the respiratory effects observed at lower concentrations in exposed humans (respiratory irritation, asthma) may be analogous to the effects seen in rats (inflammatory lung lesions, increases in inflammatory markers in BAL fluid, etc.).

In summary, the evidence supports a determination that TMBs are respiratory toxicants following inhalation exposure, based on consistency and coherency of effects observed in humans and animals, biological plausibility, observed exposure-response relationships, and evidence drawn from related compounds such as benzene, toluene, and xylene.

### 1.2.3. Reproductive and Developmental Effects

There are no studies in humans that investigated the reproductive or maternal toxicity of the TMB isomers by any route of exposure. Maternal toxicity in the form of decreased corrected body weight (i.e., maternal body weight minus the weight of the gravid uterus) was observed in Sprague-Dawley rat dams following inhalation exposure during gestation to 1,2,4-TMB or 1,3,5-TMB (Saillenfait et al., 2005) (Table 1-5; Figure 1-3). Dams exposed to 2,952 mg/m$^3$ 1,2,4-TMB gained only 86% of the weight gained by control animals, whereas dams exposed to 2,952 mg/m$^3$ 1,3,5-TMB gained only 70% of the weight gained by controls. Decreased maternal food consumption (across gestational days [GDs] 6–21) was also observed at ≥2,952 mg/m$^3$ 1,2,4-TMB (88–83%) and ≥1,476 mg/m$^3$ 1,3,5-TMB (92–75%). Maternal toxicity was also observed following exposure of CD-1 mice to the C9 fraction. Forty-four percent of dams died after exposure to 1,500 ppm C9 (4,059 mg/m$^3$ TMB isomers). Maternal body weights were significantly decreased at all exposure concentrations, and body weight gains were significantly decreased 13% at 500 ppm and 38% at 1,500 ppm C9 (1,353 and 4,059 mg/m$^3$ TMB isomers, respectively). Clinical observations of dams revealed some evidence of gross neurobehavioral toxicity including abnormal

gait (18 animals), labored breathing (9 animals), weakness (7 animals), circling (8 animals), and ataxia (8 animals).

There are no studies in humans that investigated the developmental toxicity of either 1,2,4-TMB or 1,3,5-TMB by any route of exposure. Developmental toxicity (reported as decreased fetal body weight) has been observed in male and female rats following gestational exposure to 1,2,4-TMB and 1,3,5-TMB on GDs 6 through 20 via inhalation for 6 hours/day (Saillenfait et al., 2005) (Table 1-6). Fetal body weights were decreased (statistically significantly) by 5–13% at concentrations >2,952 mg/m$^3$ of 1,2,4-TMB and 1,3,5-TMB. No adverse effects were noted on embryo/fetal viability, and no increase in skeletal, visceral, or external morphology (i.e., teratogenesis) was observed up to the highest concentrations for either isomer. Studies on the developmental or reproductive effects of 1,2,3-TMB by any route of exposure were not available. Developmental toxicity was also observed in mice exposed gestationally to the C9 aromatic fraction (>55% TMB isomers) on GDs 6–15 via inhalation for 6 hours/day (Mckee et al., 1990). Exposure to 1,500 ppm C9 (approximately 4,059 mg/m$^3$ TMBs) resulted in statistically significant decreases in live fetuses per litter, increases in post-implantation loss, and increases in the incidences of cleft palate and unossified sternebrae (#5 and/or 6). No other evidence of teratogenicity was observed. Fetal body weights (sex not reported) were statistically significantly decreased 7 and 34% following exposure to 500 ppm C9 (1,353 mg/m$^3$ TMB isomers) and 1,500 ppm C9 (4,059 mg/m$^3$ TMBs), respectively. Developmental toxicity was also observed in a study investigating the effects of multiple alkylbenzenes (benzene, toluene, xylene, ethylbenzene, Aromatol) derivatives in several species (rats, mice, rabbits) (Ungvary and Tatrai, 1985). In rats, ethylbenzene was observed to increase dead/resorbed fetuses (≥600 mg/m$^3$), decrease fetal weights (2,400 mg/m$^3$), and increase "skeletal retarded fetuses" (≥600 mg/m$^3$). Xylene and Aromatol elicited similar results, but xylene was seemingly more potent at inducing "skeletal retarded fetuses" (≥250 mg/m$^3$) compared to either ethylbenzene or Aromatol (≥600 mg/m$^3$). In mice, benzene and xylene induced weight- and skeletal-retarded fetuses at ≥500 mg/m$^3$, toluene induced these effects at 1,000 mg/m$^3$, and Aromatol did not induce these effects at any dose. Ethylbenzene, xylene, and Aromatol all increased the rate of malformations in rats and mice. In rabbits, benzene, toluene, and xylene reduced fetal weights at 500 mg/m$^3$, and all solvents resulted in spontaneous abortion at 1,000 mg/m$^3$.

No studies were available that investigated the reproductive toxicity of any TMB isomer in humans or animals. Mckee et al. (1990) investigated the reproductive toxicity of the C9 fraction in a multi-generational study of CD rats exposed to 100, 500, or 1,500 ppm C9 fraction (271, 1,353, or 4,059 mg/m$^3$ TMB isomers). No pathological lesions in the reproductive organs were noted in $F_0$ generation animals (or in any $F_1$, $F_2$, or $F_3$ animals). In the $F_0$ generation, there were no observed alterations in female or male fertility, number of females delivering a litter, or litter size at birth. However, there was a small, but not statistically significant, increase in time necessary for successful mating. No differences in $F_1$ postnatal survival were observed. Male fertility (number of fertile males/number of mated males) was significantly decreased at 1,500 ppm (4,059 mg/m$^3$

TMBs) in the $F_1$ generation. There were statistically significant reductions in the number of live $F_2$ offspring delivered per litter and the percentage of live $F_2$ births. $F_2$ generation birth weights were also decreased, but not significantly. The authors reported that among mated $F_1$ females, mating of 24 females (6 in the control group, 8 at 100 ppm [271 mg/m$^3$ TMB isomers], 1 at 500 ppm [1,353 mg/m$^3$ TMB isomers], and 9 at 1,500 ppm [4,059 mg/m$^3$ TMB isomers]) was not confirmed, and exposure was carried out until delivery, rather than being terminated on GD 20. When the dams were analyzed as separate groups, the $F_2$ litter size was only statistically significantly decreased in litters delivered from the dams that were exposed until delivery. In dams with exposure termination on GD 20, $F_2$ litter size was slightly, but not significantly, decreased. The percentage of $F_3$ live births was decreased in both groups of dams; among the dams that were exposed until delivery, pup survival was still decreased at postnatal day (PND) 4. There were no observed effects on the mean number of live $F_3$ births or postnatal survival. Birth weights of the $F_3$ generation were statistically significantly decreased (5%) in the 1,500 ppm group (4,059 mg/m$^3$ TMB isomers).

Throughout the multi-generational study, increased mortality was observed in the 1,500 ppm C9 (4,059 mg/m$^3$ TMB isomers) exposure group: 7 $F_0$ females, 6 $F_1$ females, and 36 and 34 $F_2$ males and females, respectively. Also, body weights were consistently decreased in exposed animals throughout the study, with decreases in postnatal weights occurring at lower doses in later generations compared to earlier generations. In the $F_0$ generation, male and female body weight gains were decreased 5–7% in the 500 ppm C9 exposure group (1,353 mg/m$^3$ TMB isomers), and decreased 5–7% (females) or 14–16% (males) in the 1,500 ppm group (4,059 mg/m$^3$ TMB isomers). Although birth weights were not decreased in the $F_1$ generations, mean body weights were decreased 12 and 21% in the 1,500 ppm (4,059 mg/m$^3$) group on PNDs 7 and 14 (respectively), and remained decreased in both males (24%) and females (23%) at PND 21. These decrements in body weight continued throughout the $F_1$ exposure period (which began on approximately PND 31 and continued for 10 weeks) with male and female body weights decreased 21–25 and 9–14% in the 1,500 ppm group (4,059 mg/m$^3$ TMB isomers). Additionally, male body weights were decreased 5–7% in males in the 500 ppm group (1,353 mg/m$^3$ TMB isomers). A similar pattern was observed in the $F_2$ generation, with decreases in body weights in the 1,500 ppm group (4,059 mg/m$^3$ TMB isomers) observed starting on PND 7 and continuing throughout the adult exposure and mating phase. Decreases in the adult $F_2$ generation body weights were more substantial compared to the $F_1$ generation: 31–38% in males and 21–30% in females. Body weights were also decreased 6–10% in both sexes in the 100 ppm group (271 mg/m$^3$ TMB isomers), and 16% in both sexes in the 500 ppm group (1,353 mg/m$^3$ TMB isomers). In the $F_3$ generation, no decreases in body weight were observed on PND 4, but body weights were decreased 14% in the 1,500 ppm group (4,059 mg/m$^3$) on PND 7, and 11 and 21% in the 500 and 1,500 ppm groups (1,353 and 4,059 mg/m$^3$ TMB isomers, respectively) on PND 14. These decrements in body weight were also observed in males (500 ppm: 10%; 1,500 ppm: 24%) and

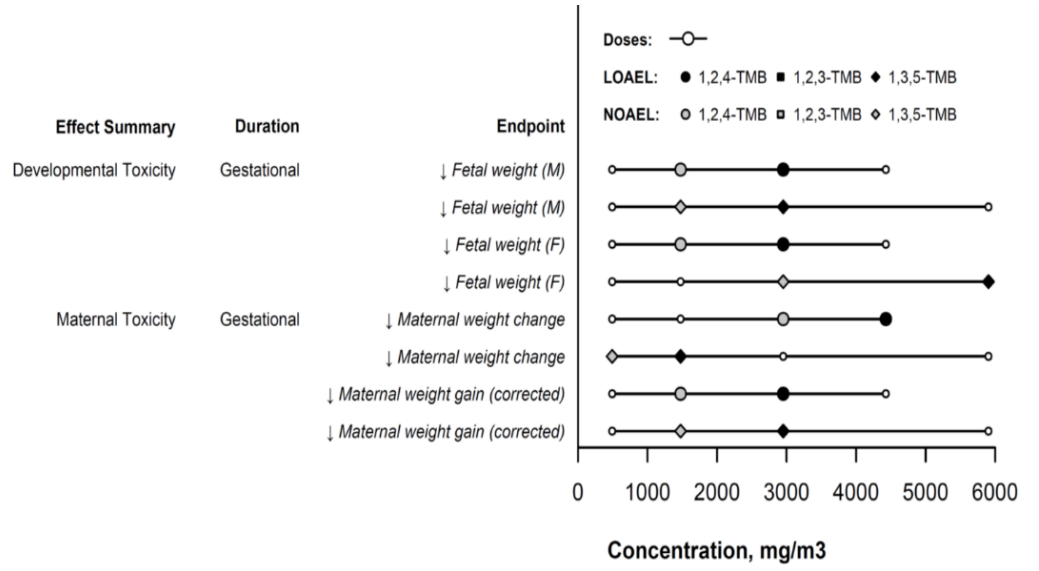
females (500 ppm: 10%; 1,500 ppm: 23%) on PND 21. Lastly, $F_1$ males and females in the 1,500 ppm group exhibited some gross signs of neurotoxicity in the form of ataxia (18 males, 23 females) and/or decreased motor activity (11 males, 8 females).

**Table 1-5. Evidence pertaining to reproductive and developmental effects of TMBs in animals—inhalation exposures**

| Study design[a] and reference | Results |
|---|---|
| ***1,2,4-TMB*** | |
| ***Developmental toxicity*** | |
| 0, 492, 1,476, 2,952, 4,428 mg/m$^3$,<br>GDs 6–20 (6 hrs/d)<br>Rat, Sprague-Dawley, female and male,<br>N = 275–342<br>Saillenfait et al. (2005), Table C-37[b] | Decreased fetal body weight of male and female fetuses.<br>*Response relative to control:*<br>Male: 0, −1, −2, −5*, −11**%<br>Female: 0, −1, −3, −5*, −12**% |
| ***Maternal toxicity*** | |
| 0, 492, 1,476, 2,952, 4,428 mg/m$^3$,<br>GDs 6–20 (6 hrs/d)<br>Rat, Sprague-Dawley, female,<br>N = 24–25 dams<br>Saillenfait et al. (2005), Table C-37 | Decreased maternal weight gain, GDs 6–15.<br>*Response relative to control:* 0, −5, −4, −11**, −27**% |
| ***1,3,5-TMB*** | |
| ***Developmental toxicity*** | |
| 0, 492, 1,476, 2,952, 5,904 mg/m$^3$,<br>GDs 6–20 (6 hrs/d)<br>Rat, Sprague-Dawley, female and male,<br>N = 217–314<br>Saillenfait et al. (2005), Table C-37 | Decreased fetal body weight of male and female.<br>*Response relative to control:*<br>Male: 0, −1, −5, −7*, −12**%<br>Female: 0, −1, −4, −6, −13**% |
| ***Maternal toxicity*** | |
| 0, 492, 1,476, 2,952, 5,904 mg/m$^3$,<br>GDs 6–20 (6 hrs/d)<br>Rat, Sprague-Dawley, female,<br>N = 24–25 dams<br>Saillenfait et al. (2005), Table C-37 | Decreased maternal weight gain, GDs 6–15.<br>*Response relative to control*: 0, 2, −13*, −30**, −46**% |
| ***C9 fraction*** | |
| ***Developmental toxicity*** | |
| 0, 100, 500, 1,500 ppm C9 fraction (approximately 0, 270, 1,353, 4,059 mg/m$^3$ TMB isomers), GDs 6–15 (6 hrs/d)<br>Mice, CD-1, female and male,<br>N = 22–27 litters<br>Mckee et al. (1990), Table C-35 | Live fetuses/litter<br>*Response relative to control:* 0, −19*, −13, −26*%<br>Post-implantation loss/dam<br>*Response relative to control:* 0, 255, 222, 478**%<br>Fetal body weight (g)<br>*Response relative to control:* 0, −1, −7*, −34**% |

| Study design[a] and reference | Results | |
|---|---|---|
| ***Maternal toxicity*** | | |
| 0, 100, 500, 1,500 ppm C9 fraction (approximately 0, 270, 1,353, 4,059 mg/m$^3$ TMB isomers), GDs 6–15 (6 hrs/d)<br>Mice, CD-1, female and male,<br>N = 28–30 litters<br>Mckee et al. (1990), Table C-35 | Decreased maternal body weight gain (GDs 6–15)<br>*Response relative to control:* 0, −13, −13*, −38*% | |
| ***Reproductive toxicity*** | | |
| 0, 100, 500, 1,500 ppm C9 fraction (approximately 0, 270, 1,353, 4,059 mg/m$^3$ TMB isomers), GDs 6–15 (6 hrs/d)<br>Rats, CD, female and male,<br>N = 28–30 litters<br>Mckee et al. (1990), Table C-35 | Male fertility index ($F_1$ generation)<br>*Response relative to control*: 0, −3, 4, −28*%<br>Litter size at birth<br>*Response relative to control*: 0, −10, −6, −30**%<br>Gestational survival index<br>*Response relative to controls*: 0, −2, −6, −13*% | |
| ***Intergenerational toxicity*** | | |
| 0, 100, 500, 1,500 ppm C9 fraction (approximately 0, 270, 1,353, 4,059 mg/m$^3$ TMB isomers), GDs 6–15 (6 hrs/d)<br>Rats, CD, female and male,<br>N = 28–30 litters<br>Mckee et al. (1990), Table C-35 | Decreased birth/pup/adult body weights, *response relative to controls* | |
| | Birth weight<br>$F_1$: 0, 2, +6, 0%<br>$F_2$: 0, 2, 0, −5%<br>$F_3$: 0, 0, 2, −5**% | Day 4 body weights<br>$F_1$: 0, 1, 4, −5%<br>$F_2$: 0, 5, 4, −2%<br>$F_3$: 0, 3, 1, −5% |
| | Day 7 body weights<br>$F_1$: 0, −3, 2, −12**%<br>$F_2$: 0, 0, 1, −12**%<br>$F_3$: 0, 1, −4, −14**% | Day 14 body weights<br>$F_1$: 0, −7, −4, −21**%<br>$F_2$: 0, −3, −2, −21**%<br>$F_3$: 0, −2, −11**, −21**% |
| | Day 21 male body weights<br>$F_1$: 0, −6, 1, −24**%<br>$F_2$: 0, −4, −3, −26%<br>$F_3$: 0, 0, −10*, −24**% | Day 21 female body weights<br>$F_1$: 0, −6, 0, −23**%<br>$F_2$: 0, −4, −3, −27** %<br>$F_3$: 0, 0, −10**, −23**% |

*Statistically significantly different from controls at $p < 0.05$.
**Statistically significantly different from controls at $p < 0.01$.
[a]In instances where authors reported exposures in ppm, EPA converted these values to mg/m$^3$. See Table P-1 for conversion factor, and individual study summary tables for ppm values. N refers to number animals/group.
[b]Tables referenced in Study Design and Reference column correspond to study summary tables in Appendix C.




Note: Solid lines represent range of exposure concentrations. All effects from Saillenfait et al. (2005). Effects in italics are from studies that reported actual exposure concentrations.

**Figure 1-3. Exposure response array of developmental effects following inhalation exposure to 1,2,4-TMB or 1,3,5-TMB.**

### *Mode-of-Action Analysis—Reproductive and Developmental Effects*

Data regarding the potential mode of action for the reproductive and developmental effects resulting from TMB inhalation exposures are not available, and the key events for TMB-induced reproductive and developmental toxicity are not established. However, evidence drawn from the literature regarding related alkylbenzene compounds provides suggestive evidence that alkylbenzene exposures modulate endocrine function and signaling (Bolden et al., 2015). Alkylbenzenes (especially benzene and toluene) are associated with endpoints such as altered menstrual cycles and sperm production, and have been shown to alter the concentrations of luteinizing hormone, follicle stimulating hormone, and testosterone in occupationally exposed populations (Bolden et al., 2015). Further, alkylbenzene-induced alterations in the concentrations of other hormones (insulin-like growth factor, thyroid hormone) and perturbation of endocrine signaling (glucocorticoid, estrogen, and progesterone) may explain observed alterations in fetal growth and development of inflammatory responses (respectively) similar to those observed in TMB studies (Bolden et al., 2015; Billionnet et al., 2011; Saillenfait et al., 2005; Korsak et al., 2000a, b).

### *Summary of Reproductive and Developmental Effects*

The database for reproductive and developmental toxicity following inhalation exposure to 1,2,4-TMB and 1,3,5-TMB is limited to one animal developmental study; no studies in humans are available. Thus, these isomers may cause developmental toxicity, although this is based on only one

study that demonstrated clear, exposure-related effects on fetal and maternal body weights. There is possibly confirmatory evidence of TMB-induced developmental and/or reproductive effects given the results of studies of the C9 fraction that observed increased fetal loss and decreased fetal weights in mice, and decreased measures of fertility in rats. Information from integrated reviews of the alkylbenzene literature provides evidence that compounds are associated with a number of reproductive and/or developmental endpoints such as decreased semen quality/sperm count (benzene, toluene), altered menstrual cycling and fecundity (benzene, toluene), miscarriage and stillbirth (benzene, toluene), malformations (benzene), and low birth weight (toluene, ethylbenzene, xylene) (Bolden et al., 2015; Webb et al., 2014). Additionally, there was suggestive evidence of intergenerational TMB-toxicity as decreases in fetal/pup/adult body weights were observed to occur at lower doses in later generations compared to earlier ones in the exposed rats. However, comprehensive reviews of the styrene (a related alkylbenzene) database indicate that styrene is likely not a reproductive or developmental toxicant (Luderer et al., 2006; Brown et al., 2000).

In summary, the evidence supports a determination that TMBs are developmental toxicants following inhalation exposure, based on biological plausibility and observed exposure-response relationships. Supportive evidence from related compounds and mixtures containing TMB isomers support this determination. Although no direct evidence exists for TMB isomers, evidence from related compounds and mixtures containing TMB isomers indicate that TMB isomers may be reproductive toxicants as well.

### 1.2.4. Hematological and Clinical Chemistry Effects

There is limited evidence in humans, and stronger evidence in animals, that exposure to TMB isomers via inhalation induces hematological toxicity and alterations in clinical chemistry parameters. Alterations in blood clotting and anemia in workers exposed to a paint solvent containing 50% 1,2,4-TMB, 30% 1,3,5-TMB, and unspecified amounts of 1,2,3-TMB (listed as possibly present) was reported by Bättig et al. (1956), as reviewed by MOE (2006); effects were observed at 295 mg/m$^3$. Studies identifying an association between occupational exposure to TMB isomers and hematological and clinical chemistry effects are limited due to an inability to attribute effects due to 1,2,3-TMB, 1,2,4-TMB, or 1,3,5-TMB individually versus those due to the other isomers or additional constituents within the solvent mixtures. For example, Gerarde (1960) suggested that the hematological effects observed in Bättig et al. (1956) may have been due to trace amounts of benzene present in the solvent mixture.

In animals, there is evidence of hematological toxicity following subchronic inhalation exposure to 1,2,4-TMB or 1,2,3-TMB and short-term inhalation exposure to 1,3,5-TMB (Table 1-6; Figure 1-4). Subchronic exposures to 1,2,4-TMB or 1,2,3-TMB have been shown to result in hematological effects and changes in serum chemistry in rats (Korsak et al., 2000a, b). In male rats exposed to 1,230 mg/m$^3$ 1,2,4-TMB or 1,2,3-TMB, RBC counts were significantly decreased 23 and 15%, respectively. The observed alterations in RBCs were concentration-dependent as determined

by trend analysis. Exposure to 1,2,4-TMB or 1,2,3-TMB did not significantly decrease RBCs in female rats, but trend analysis demonstrated that decreases in RBC counts in female rats exposed to 1,2,3-TMB were concentration dependent, with a maximum decrease of 9% at 1,230 mg/m$^3$. RBCs in both sexes were observed to still be depressed relative to controls 2 weeks following termination of exposure to both isomers, but these decreases were not statistically significant.

WBC counts were significantly increased 80% in male rats and increased 30% (not statistically significant) in female rats exposed to 1,230 mg/m$^3$ 1,2,4-TMB. After a 2-week follow-up after termination of exposure, WBC counts had returned to normal in female rats and were slightly depressed (18%) in male rats. WBC numbers were unchanged in male rats exposed to 1,2,3-TMB, but were increased (not statistically significant) 22% in female rats exposed to 1,230 mg/m$^3$. Two weeks following termination of exposure, WBC counts in male and female rats had fallen to roughly 60% of controls.

Significant decreases in reticulocytes (71% decrease relative to controls) and clotting time (37% decrease relative to controls) were observed in female rats exposed to 1,230 and 492 mg/m$^3$ 1,2,4-TMB, respectively. Both of these effects were concentration-dependent across the entire range of concentrations as determined by trend-analysis; animals fully recovered within 2 weeks after termination of exposure. Reticulocyte numbers were statistically significantly increased 60% in male rats exposed to 1,230 mg/m$^3$ 1,2,3-TMB, with reticulocyte numbers even further increased (150%) 2 weeks following the termination of exposure. Reticulocyte numbers in females exposed to 1,2,3-TMB were significantly increased 77 and 100% at 123 and 492 mg/m$^3$, respectively, and increased 69% (not statistically significant) at 1,230 mg/m$^3$. Reticulocyte numbers were still increased in males and females 2 weeks after the termination of exposure to 1,2,3-TMB. Segmented neutrophils were statistically significantly decreased 29% in male rats exposed to 1,230 mg/m$^3$ 1,2,3-TMB; statistically significant decreases of 29 and 48% were observed in female rats exposed to 492 and 1,230 mg/m$^3$ 1,2,3-TMB. Lymphocytes were statistically increased 11 and 15% in male and female rats exposed to 1,230 mg/m$^3$, respectively. Numbers of segmented neutrophils and lymphocytes returned to control values 2 weeks after termination of exposure. In a study investigating the hematological toxicity of the C9 aromatic fraction (~45% TMB isomers), few consistent trends were reported for any of the hematological parameters investigated (Clark et al., 1989). A decrease (0.42 versus 0.40) in osmotic fragility (50%) was noted in rats exposed to 450, 900, and 1,800 mg/m$^3$ C9 fraction (corresponding to 203, 405, and 810 mg/m$^3$ TMB isomers, respectively) for 12 months. Additional effects included increased WBCs and absolute lymphocytes in males exposed to 1,800 mg/m$^3$ C9. Another study investigating a complex solvent mixture (white spirit) containing TMB isomers (albeit at a much lower proportion of the mixture, total C9 fraction 8–11%, TMB content not reported) reported alterations in some hematological and clinical parameters (decreased packed cell volume [PCV], decreased RBCs, increased mean cell volume [MCV], and increased AP and aspartate aminotransferase [AST]) (Carrillo et al., 2014).

Exposure to TMB isomers was also observed to have an effect on clinical chemistry markers that possibly indicate hepatic injury. Sorbitol dehydrogenase (SDH) was increased at ≥123 mg/m$^3$ in male rats exposed to 1,2,4-TMB (18–23% relative to controls) and at 1,230 mg/m$^3$ in male rats exposed to 1,2,3-TMB (69% relative to controls) (Korsak et al., 2000a, b). However, the increases following exposure to 1,2,4-TMB were not concentration-dependent. SDH activity was also higher in female rats exposed to 1,2,4-TMB (19–23% relative to controls), but the increases in activity were not significantly higher when compared to controls. SDH activity was not affected in female rats exposed to 1,2,3-TMB. Alanine aminotransferase (ALT) was decreased (23% relative to controls) and AP was increased (42–45% relative to controls) at 1,230 and ≥492 mg/m$^3$ (respectively) in female rats exposed to 1,2,3-TMB. Absolute liver weights were only observed to increase (9%) in male rats exposed to 1,230 mg/m$^3$ 1,2,3-TMB, and no histopathological changes were observed in either sex exposed to 1,2,3-TMB or 1,2,4-TMB. Therefore, the adversity of the observed changes in clinical chemistry parameters is unclear. In rats exposed to the C9 fraction for 12 months, alterations in clinical chemistry parameters were generally mild, with females exposed to 1,800 mg/m$^3$ C9 fraction exhibiting increased sodium and decreased albumin (Clark et al., 1989). The only clinical chemistry effect in exposed males was increased creatinine at 1,800 mg/m$^3$.

An increase (30% relative to controls) in AST, but no other substantial hematological effects, was observed in rats 14 days following short-term exposure (6 hours/day, 6 days/week for 5 weeks) (Wiglusz et al., 1975b; Wiglusz et al., 1975a). The adversity of AST is uncertain given the lack of a clear pattern in temporality (effects at some days post-exposure, but not others) and the lack of accompanying liver histopathology.

Acute inhalation exposures of male Wistar rats to 1,500–6,000 mg/m$^3$ 1,3,5-TMB for 6 hours did not result in substantial effects on hemoglobin or RBC or WBC count (Wiglusz et al., 1975b). However, the number of segmented neutrophilic granulocytes was increased in 1,3,5-TMB-exposed rats up to 28 days following exposure (statistics not reported). The greatest increase in granulocyte numbers (100%) was observed the day of exposure and 1 day following exposure in rats exposed to 6,000 mg/m$^3$, although attenuation was seen 7–28 days following exposure, possibly indicating induction of metabolizing enzymes or saturation of toxicity pathways. Investigation of clinical chemistry parameters in rats acutely exposed to 300–3,000 mg/m$^3$ for 6 hours did not reveal any consistent pattern in the levels of AST or ALT, although AP was statistically increased 84% in rats 7 days following exposure to 3,000 mg/m$^3$ (Wiglusz et al., 1975a).

Slight alterations in clinical chemistry parameters and differential WBC counts were also observed in rats following subchronic, oral exposure to 1,3,5-TMB (Table 1-7; Figure 1-5) (Adenuga et al., 2014; Koch Industries, 1995b). While no hematological parameters (i.e., RBC counts, hematocrit) were observed to differ between exposed rats and controls, the number of monocytes increased (100–200% increase) in male rats exposed to ≥200 mg/kg-day 1,3,5-TMB. Additionally, a number of clinical chemistry parameters were altered in exposed rats. In female rats exposed to

600 mg/kg-day, sodium and chloride levels were statistically significantly decreased (2.3 and 2.7%, respectively) relative to controls, and cholesterol and phosphorus were statistically significantly increased (41 and 23%, respectively). In male rats, exposure to 600 mg/kg-day resulted in a significant decrease (19%) in glucose levels and significant increases in phosphorus levels and AP activity (17 and 46%, respectively). In a related, preliminary study (Koch Industries, 1995a), hematological and clinical chemistry effects were also observed following 14 days of oral exposure. Female Sprague-Dawley rats exposed to either 150 or 600 mg/kg-day 1,3,5-TMB had increased cholesterol levels, and high-dose males exhibited increased WBC counts with corresponding increased neutrophil and lymphocyte numbers.

**Table 1-6. Evidence pertaining to hematological and clinical chemistry effects of TMBs in animals—inhalation exposures**

| Study design[a] and reference | Results |
|---|---|
| ***1,2,4-TMB*** | |
| ***Hematological toxicity*** | |
| 0, 123, 492, 1,230 mg/m$^3$, 90 d (6 hrs/d, 5 d/wk)<br>Rat, Wistar, female and male, N = 10<br>Korsak et al. (2000a), Table C-30[b] | Decreased RBCs in males only<br>*Response relative to control:* 0, −1, −15, −23**%<br>(recovery = −24%)<br>Increased WBCs in males only<br>*Response relative to control:* 0, 2, 4, 80**%<br>(recovery = −18%)<br>Decreased reticulocytes in females only<br>*Response relative to control:* 0, −51, −49, −71*%<br>(recovery = 65%)<br>Decreases in clotting time in females only<br>*Response relative to control:* 0, −23, −37**, −27*%<br>(recovery = 60%) |
| ***Clinical chemistry effects*** | |
| 0, 123, 492, 1,230 mg/m$^3$, 90 d (6 hrs/d, 5 d/wk)<br>Rat, Wistar, female and male, N = 10<br>Korsak et al. (2000a), Table C-30 | Non-monotonic increases in SDH in males only<br>*Response relative to control:* 0, 73**, 74*,73**% |

| Study design[a] and reference | Results |
|---|---|
| ***1,2,3-TMB*** | |
| ***Hematological toxicity*** | |
| 0, 123, 492, 1,230 mg/m$^3$, 90 d (6 hrs/d, 5 d/wk)<br>Rat, Wistar, female and male, N = 10<br>Korsak et al. (2000b), Table C-31 | Decreased RBCs in males only<br>*Response relative to control:* 0, 8, 6, −15*% (recovery = −9%)<br>Decreased segmented neutrophils in males and females.<br>*Response relative to control:*<br>*Males:* 0, 2, −17, −29*% (recovery = 11% increase)<br>*Females:* 0, −15, −29*, −48*% (recovery = 15% decrease)<br>Increased lymphocytes in males and females.<br>R*esponse relative to control:*<br>*Males:* 0, 1, 6, 11**% (recovery = 11% decrease)<br>*Females:* 0, 6, 10, 15**% (recovery = 3% increase)<br>Increased reticulocytes in males and females (non-monotonic).<br>*Response relative to control:*<br>*Males:* 0, −25, 36, 61*% (recovery = 146**% increase)<br>*Females:* 0, 77*, 100**, 69% (recovery = 162**% increase) |
| ***Clinical chemistry effects*** | |
| 0, 123, 492, 1,230 mg/m$^3$, 90 d (6 hrs/d, 5 d/wk)<br>Rat, Wistar, female and male, N = 10<br>Korsak et al. (2000b), Table C-31 | Decreased ALT in females only<br>*Response relative to control:* 0, −1, −6, −23*%<br>Increased AP in females only.<br>*Response relative to control:* 0, 20, 45*, 42*%<br>Increased SDH in males only<br>*Response relative to control:* 0, 44, 56, 69*% |
| ***1,3,5-TMB*** | |
| ***Hematological toxicity*** | |
| 1,500, 3,000, 6,000 mg/m$^3$, 6 hrs<br>Samples collected 0, 1, 7, 14, and 28 d post-exposure<br>Rat, Wistar, male, N = 5−8<br>Wiglusz et al. (1975b), Table C-44 | Increased segmented neutrophilic granulocytes (1−28 d post-exposure)<br>*Response relative to control:*<br>*Day 0:* 0, 59, 118, 95%<br>*Day 1:* control response not reported<br>*Day 7:* control response not reported<br>*Day 14:* 0, 15, 184, 94%<br>*Day 28:* 0, −20, 124, 1% |
| ***Clinical chemistry effects*** | |
| 3,000 mg/m$^3$, 5 weeks (6 hrs/day, 6 d/wk)<br>Samples collected 1, 3, 7, 14, and 28 d during exposure<br>Rat, Wistar, male, N = 6<br>Wiglusz et al. (1975a), Table C-45 | Increased AST on d 14<br>*Response relative to control (d 14):* 12*%<br>Increased AP on d 7 post-exposure<br>*Response relative to control (on d 7):* 0, −0.1, 0.03, 84*% |

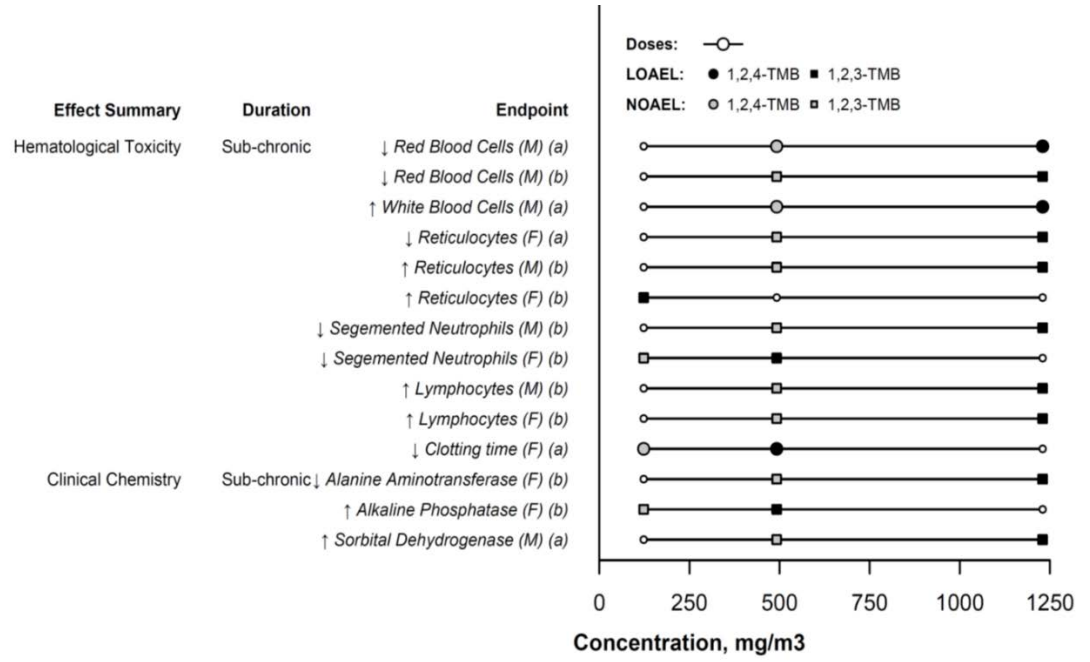
| Study design[a] and reference | Results |
|---|---|
| ***C9 fraction*** | |
| ***Hematological toxicity*** | |
| 0, 450, 900, 1,800 C9 fraction (approximately 0, 203, 405, 810 mg/m$^3$ TMB isomers), 12 mo (5 d/wk, 6 hrs/d)<br>Rats, Wistar, male, N = 50<br>Clark et al. (1989), Table C-20 | Decreased osmotic fragility<br>*Response relative to controls*: 0, −5, −5, −5%<br>Increased WBCs<br>*Response relative to controls*: 0, −3, 9, 27%<br>Increased absolute lymphocytes<br>*Response relative to controls*: 0, 5, −5, 29*% |
| ***Clinical chemistry effects*** | |
| 0, 450, 900, 1,800 C9 fraction (approximately 0, 203, 405, 810 mg/m$^3$ TMB isomers), 12 mo (5 d/wk, 6 hrs/d)<br>Rats, Wistar, male and female, N = 50<br>Clark et al. (1989), Table C-20 | Increased creatinine (males)<br>*Response relative to controls*: 0, 0, 7, 9*%<br>Increased sodium (females)<br>*Response relative to controls*: 0, 0, 1, 1*%<br>Decreased albumin (females)<br>*Response relative to controls*: 0, 1, −5, −9*% |

*Statistically different from controls at $p < 0.05$.
**Statistically different from controls at $p < 0.01$.
[a]In instances where authors reported exposures in ppm, EPA converted these values to mg/m$^3$. See Table P-1 for conversion factor, and individual study summary tables for ppm values. N refers to number animals/group.
[b]Tables referenced in the *Study design and reference* column correspond to study summary tables in Appendix C.

**Table 1-7. Evidence pertaining to hematological and clinical chemistry effects of TMBs in animals—oral exposures**

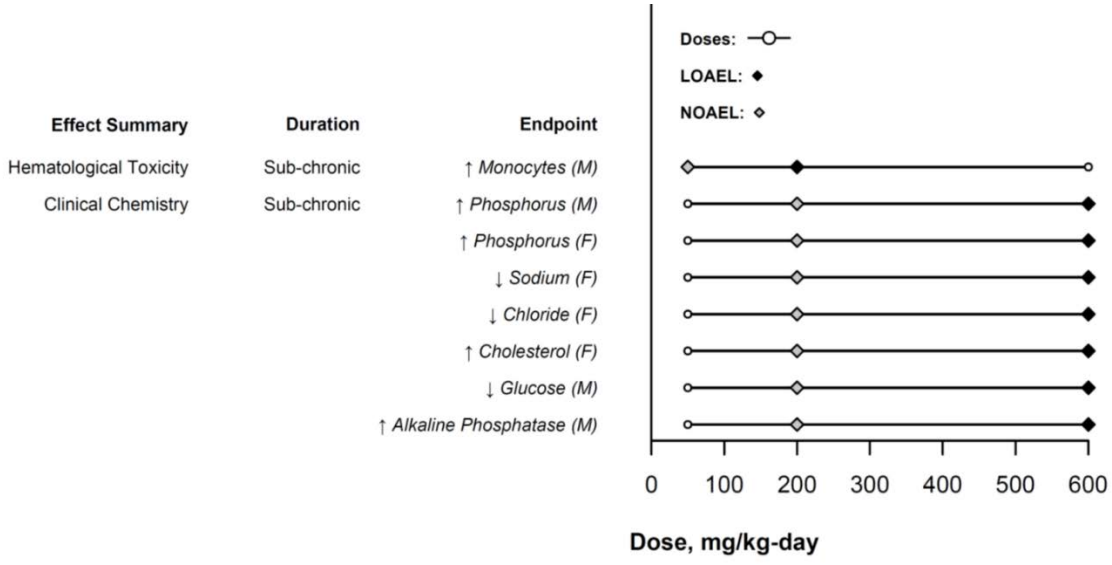
| Study design[a] and reference | Results |
|---|---|
| ***1,3,5-TMB*** | |
| ***Hematological toxicity*** | |
| 0, 50, 200, 600 mg/kg-day, 90 d (once daily, 5 d/wk)<br>Rat, Sprague-Dawley, female and male, N = 10<br>Adenuga et al. (2014); Koch Industries (1995b), Tables C-26[b] and C-17 | Increased monocyte levels in males only<br>*Response relative to control:*<br>Male: 0, 100, 200*, 100*% (recovery = 100% increase) |
| ***Clinical chemistry effects*** | |
| 0, 50, 200, 600 mg/kg-day, 90 d (once daily, 5 d/wk)<br>Rat, Sprague-Dawley, female and male, N = 10<br>Adenuga et al. (2014); Koch Industries (1995b), Tables C-26[b] and C-17 | Increased phosphorus levels in males and females<br>*Response relative to control:*<br>Male: 0, 3, 8, 17*% (recovery = 11% decrease)<br>Female: 0, 0, 5, 23*% (recovery = 13% decrease)<br>Decreased sodium levels in females only<br>*Response relative to control:* 0, 0, 0, −2*%<br>(recover = 1% decrease)<br>Decreased chloride levels in females only<br>*Response relative to control:* 0, 0, 0, −3*%<br>(recovery = 1% increase)<br>Increased cholesterol levels in females only<br>*Response relative to control:* 0, −3, 7, 41*%<br>(recovery = 21% decrease)<br>Decreased glucose levels in males only<br>*Response relative to control:* 0, −10, −9, −19*%<br>(recovery = 12% increase)<br>Increased AP activity in males only<br>*Response relative to control:* 0, 5, 13, 46*%<br>(recovery = 28% decrease) |

*Statistically different from controls at $p < 0.05$.
**Statistically different from controls at $p < 0.01$.
[a]N refers to number animals/group.
[b]Tables referenced in the *Study design and reference* column correspond to study summary tables in Appendix C.




Note: Solid lines represent range of exposure concentrations. (a) Korsak et al. (2000a); (b) Korsak et al. (2000b). Effects in italics are from studies that provided actual exposure concentrations as measured by gas chromatography.

**Figure 1-4. Exposure response array of hematological and clinical chemistry effects following inhalation exposure to 1,2,4-TMB or 1,2,3-TMB.**




Note: Solid lines represent range of exposure concentrations. All effects from Adenuga et al. (2014). Effects in italics are from studies that provided actual exposure concentrations as measured by gas chromatography.

**Figure 1-5. Exposure response array of hematological and clinical chemistry effects following oral exposure to 1,3,5-TMB.**

***Mode-of-Action Analysis—Hematological and Clinical Chemistry Effects***

The mode of action for TMB-induced hematological and clinical chemistry effects has not been established. Increased SDH and AP activity are both markers for hepatic injury (Adenuga et al., 2014; Ramaiah, 2007) and therefore, underlying hepatotoxicity could explain their increase in rats exposed to 1,2,4-TMB or 1,2,3-TMB via inhalation, or 1,3,5-TMB via oral ingestion. However, absolute and relative liver weights were not observed to increase with inhalation exposure to 1,2,4-TMB (Korsak et al., 2000a), and relative liver weights were only observed to increase 9% over controls in male rats exposed to 1,230 mg/m$^3$ 1,2,3-TMB. Increases in relative liver weights were also observed males and females exposed orally to 1,3,5-TMB. However, in studies that observed liver weight increases, microscopic histopathological analyses of the liver did not demonstrate any observable changes in either sex following exposure to TMB isomers. Therefore, the adversity of the observed changes in clinical chemistry parameters is unclear. Similarly, although increased cholesterol levels could also indicate hepatic dysfunction, the lack of gross or histopathological lesions in animals orally exposed to 1,3,5-TMB calls into question the adversity of this particular finding. The increases in WBC counts in exposed animals could be secondary to the observed respiratory irritative and inflammatory effects of 1,2,4-TMB exposure in Korsak et al. (2000a) and Korsak et al. (1997).

***Summary of Hematological and Clinical Chemistry Effects***

Hematological and clinical chemistry toxicity was consistently observed following inhalation and oral exposure to TMBs based on coherent evidence in humans and animals. The information regarding hematological toxicity in humans is limited to one study involving exposure to a complex VOC mixture containing both 1,2,4-TMB and 1,3,5-TMB (Bättig et al. (1956), as reviewed in MOE (2006) and Bättig et al. (1958)). Although this study reported hematological effects coherent with those observed in animal studies (alterations in clotting and anemia), exposure was to a mixture of TMB isomers and other VOCs. Therefore, it is impossible to attribute the effects to any TMB isomer. There is strong and consistent evidence of hematological effects in male and female Wistar rats following inhalation exposure (Korsak et al., 2000a, b) that are roughly analogous to those observed in humans. Additionally, there is some evidence of hematological and clinical chemistry effects in male and female Sprague-Dawley rats following oral exposure (Adenuga et al., 2014; Koch Industries, 1995b). Given the observation of increased WBC counts, decreased RBC counts, and alteration of clinical chemistry parameters following exposure to all TMB isomers, it is possible that these effects are markers of underlying TMB-induced hepatotoxicity. However, no study that investigated histopathological changes in the liver observed any changes that might indicate toxicity or injury to that organ. Similarly, the observed alterations in WBC counts might be a biomarker of effect for the consistently reported respiratory effects, most of which are observed to be irritative or inflammatory in nature. An integrated review of the health

effects literature for related compounds indicates that exposure to benzene is associated with decreased hemoglobin, hematocrit, and RBC counts (Bolden et al., 2015).

In summary, the evidence supports a determination that 1,2,4-TMB and 1,2,3-TMB result in hematological toxicity following inhalation exposure, based on consistency and coherency of effects across species (human and rats) and the observation of similar effects following exposure to the related compound benzene.

### 1.2.5. General Toxicity

Decreased body weight as a marker of general systemic toxicity has not been observed consistently in TMB studies. For instance, no study investigating the toxicity of individual TMB isomers in adult animals has observed decreased body weight following inhalation or oral administration to TMB (Adenuga et al., 2014; Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Korsak et al., 2000a; Wiaderna et al., 1998; Gralewicz et al., 1997b; Korsak and Rydzyński, 1996). However, decreased body weight has been observed in pregnant dams as a marker of maternal toxicity following gestational exposure to 1,2,4-TMB or 1,3,5-TMB via inhalation (Saillenfait et al., 2005). Similarly, decreased fetal weight has been observed as a marker of developmental toxicity in animals exposed to either 1,2,4-TMB, 1,3,5-TMB, or the C9 fraction (Saillenfait et al., 2005; Mckee et al., 1990). A possible cumulative intergenerational effect on postnatal body weight was observed in a multi-generational reproductive toxicity study (Mckee et al., 1990).

Decreased body weight gains in $F_0$ adults (exposure beginning prior to mating) were observed at 500 and 1,500 ppm C9 fraction (approximately 1,353 and 4,059 mg/m$^3$ TMB isomers) (Mckee et al., 1990). Although birth weights were not decreased in the $F_1$ generation, decreases in body weights were observed in the 1,500 ppm C9 (4,059 mg/m$^3$ TMB isomers) group beginning at PND 7 and continuing through adulthood; a similar pattern was observed in $F_2$ animals. In the $F_3$ generation, birth weight and PND 7 body weight were decreased at 1,500 ppm (4,059 mg/m$^3$ TMB isomers). Beginning on PND 14, body weight was decreased in both the 500 and 1,500 ppm exposure groups (1,353 and 4,059 mg/m$^3$ TMB isomers). Decreases in adult body weights have been observed in other C9 fraction toxicity studies. Body weights were slightly decreased relative to control during the first 4 weeks of a 12-month study in male rats exposed to 1,830 mg/m$^3$ C9 and females exposed to 970 mg/m$^3$ and during the first 12 weeks of exposure in females exposed to 1,830 mg/m$^3$ (Clark et al., 1989). In a 13-week neurotoxicity study (Douglas et al., 1993), animals in the high-exposure group (i.e., 1,500 ppm C9 [4,059 mg/m$^3$ TMB isomers]) exhibited statistically decreased body weights at every time point during exposure; animals in the 500 ppm group (1,353 mg/m$^3$ TMB isomers) had decreased body weights early during exposure, with a statistically significant decrease at week 4. However, by the end of the exposure period, these animals weighed more than controls. Decreases in body weight were also observed in male and female rats exposed to white spirit containing 8–11% C9 aromatics (proportion of individual TMB isomers not specified) (Carrillo et al., 2014).

Other markers of possible systemic toxicity included alternations in organ weight in multiple organs, most commonly the liver. Relative liver weights were increased in male and female rats following oral exposure to 600 mg/kg-day 1,3,5-TMB (Adenuga et al., 2014), male rats exposed to 1,230 mg/m$^3$ 1,2,3-TMB (Korsak et al., 2000b), and male and female rats exposed to ≥4,000 mg/m$^3$ white spirit (Carrillo et al., 2014). Absolute liver weight was also increased in male rats exposed to 1,800 mg/m$^3$ C9 fraction (Clark et al., 1989). Possible evidence of hepatic injury was further demonstrated by the evidence of alterations in clinical chemistry parameters in some studies. For example, increased AP, SDH, and cholesterol were all observed in treated animals (Adenuga et al., 2014; Carrillo et al., 2014; Korsak et al., 2000a, b). However, no indications of hepatic toxicity was apparent in microscopic, histopathological examinations. Given this observation, it is most likely that the changes in liver weight are not markers of adversity, but rather markers of adaptive and compensatory changes. Alterations in spleen, kidney, and heart weights were also sporadically noted (Carrillo et al., 2014; Korsak et al., 2000a, b; Clark et al., 1989), but as with the liver, gross and histopathological examinations revealed no consistent treatment-related lesions.

### 1.2.6. Carcinogenicity

There are no studies in humans that investigated the carcinogenic potential of the TMB isomers by any route of exposure. One animal study was identified that investigated the association of chronic oral exposure (via gavage) to 1,2,4-TMB and cancer endpoints (Maltoni et al., 1997). Male and female Sprague-Dawley rats were exposed to a single dose of 800 mg/kg-day of 1,2,4-TMB in olive oil by stomach tube for 4 days/week starting at 7 weeks of age. Exposures were terminated at the end of 104 weeks (i.e., at 111 weeks of age) and the animals were kept under observation until natural death. The authors reported that chronic oral exposure to 1,2,4-TMB resulted in an "intermediate" reduction of survival in male rats and a "slight" reduction in females (no quantitative information on survival was reported). A slight increase in total malignant tumors in both sexes of rats was observed, with the incidence of head cancers being specifically increased in male rats. The predominant type of head cancer identified was neuroesthesioepithelioma, which arises from the olfactory neuroepithelium and is normally rare in Sprague-Dawley rats. Other head cancers observed included those in the Zymbal gland, ear duct, and nasal and oral cavities. No tests of statistical significance were reported for these data. The carcinogenicity of the C9 fraction was investigated in rats exposed to C9 for 12 months (Clark et al., 1989). Although there were no treatment-related increases in tumors at the end of the exposure period, some sporadic tumors were noted: one leiomyoma of the left uterine horn (female, 1,800 mg/m$^3$), one lymphoma of the spleen (male, 1,800 mg/m$^3$), and one glioblastoma of the cerebellum (male, 450 mg/m$^3$). A number of pituitary adenomas were observed in both sexes in all exposure groups, including the control group.

Some evidence exists that 1,2,4-TMB can be used as a biomarker for cancer incidence. 1,2,4-TMB was detected as frequently, and in some cases more frequently, in the urine of cancer

patients (breast, colorectal, lymphoma, and leukemia) compared to noncancer controls (Silva et al., 2012; Silva et al., 2011). However, the total amount of 1,2,4-TMB recovered in the patients' urine was consistently greater by 38–99% compared to controls. Neither 1,2,3-TMB nor 1,3,5-TMB, nor any metabolites of any of the TMB isomers, were observed to be increased in cancer patients relative to controls. While this evidence is suggestive of an association between 1,2,4-TMB and the presence of various tumor types, the study's cross-sectional nature cannot support a firm conclusion that 1,2,4-TMB was a potential cause of the particular cancers under investigation.

Janik-Spiechowicz et al. (1998) investigated the genotoxicity of TMB isomers by measuring three genotoxic endpoints: mutation frequency in bacteria, micronucleus formation in mice, and sister chromatid exchanges (SCEs) in mice. Neither 1,2,4-TMB nor 1,3,5-TMB induced gene mutations in any *Salmonella typhimurium* strain tested (TA102, TA100, TA98, and TA97a). However, 1,2,3-TMB induced gene mutations in all four strains in the absence of rat S9 fraction. When cells were incubated in the presence of S9, 1,2,3-TMB did not induce gene mutation, possibly indicating that 1,2,3-TMB itself is the primary mutagen. No isomer induced the formation of micronuclei in Imp:BALB/c mice following i.p. injection. Males in the high-dose groups for 1,2,4-TMB and 1,3,5-TMB, but not 1,2,3-TMB, exhibited a statistically significant reduction in the ratio of polychromatic erythrocytes to normochromatic erythrocytes, indicating bone marrow cytotoxicity. All three isomers significantly increased the frequency of SCEs in Imp:BALB/c mice following i.p. injection, with 1,2,4-TMB eliciting the more significant response. These results appear to have occurred at doses that did not induce significant bone marrow cytotoxicity.

Schreiner et al. (1989) assessed the mutagenic potential of the C9 fraction (total TMB content = 55.05%) via multiple in vitro and in vivo assays. In a bacterial mutagenicity assay, five *S. typhimurium* test strains (TA98, TA100, TA1535, TA1537, and TA1538) were exposed to either negative controls (DMSO), positive controls, or to 0.0025–0.50 µL/plate C9 fraction in the presence or absence of the S9 microsomal mixture. There was no evidence that the C9 fraction induced gene mutations with or without S9 activation in any *S. typhimurium* strain up to the highest test concentration, at which signs of cellular toxicity became apparent. Multiple in vitro assays in Chinese hamster ovary (CHO) cells were similarly negative in the absence and presence of the S9 microsomal mixture. Mutation frequencies were not increased in CHO cells exposed to the C9 fraction for 4 hours after a 7-day incubation period. Neither SCEs nor chromosomal aberrations were increased in CHO cells at any concentration of the C9 fraction (up to concentrations of 90.2 µg/mL). In order to investigate the potential in vivo mutagenicity of the C9 fraction, Sprague-Dawley rats (30 per exposure group, 15 male and 15 female) were exposed via inhalation to 0, 150, 500, or 1,500 ppm C9 fraction for 6 hours on 5 consecutive days. Following the termination of exposure, 10 rats from each treatment group were sacrificed at 6, 24, and 48 hours, and their bone marrow was examined for chromosome/chromatid aberrations. No induction of chromosomal/ chromatid aberrations was observed at any exposure concentration in animals sacrificed any time

point. In general, the results of Schreiner et al. (1989) indicate that, as tested, the C9 fraction did not induce in vitro or in vivo mutagenicity in multiple assays.

In summary, very little genotoxicity data are available on individual isomers of TMBs. Janik-Spiechowicz et al. (1998) observed varying results in the Ames mutation assay in Salmonella, with 1,2,3-TMB, but not 1,2,4-TMB or 1,3,5-TMB, inducing gene mutations. Results for the in vivo assays for micronucleus and SCE formation were consistent across isomers: TMB isomers were observed to induce SCEs, but not micronuclei, in mouse bone marrow cells. Increased frequency of SCEs indicates that DNA damage occurred as a result of exposure to these isomers, but it does not provide a specific indication of mutagenic potential, as there is no known mechanistic association between SCE induction and a transmissible genotoxic effect. A similar lack of evidence of genotoxicity is provided by Schreiner et al. (1989) in that the C9 fraction was observed to not induce increased mutation frequencies, SCEs, or chromosomal aberrations. With only one isomer (1,2,3-TMB) demonstrating a positive result for gene mutation and positive SCE results for all three isomers, there is currently inadequate evidence to conclude that any isomer is directly genotoxic.

### 1.2.7. Similarities among TMB Isomers Regarding Observed Inhalation and Oral Toxicity

In the existing toxicological database for 1,2,3-TMB, 1,2,4-TMB, and 1,3,5-TMB, important similarities have been observed in the potency and magnitude of effect resulting from exposure to these three isomers in male and female Wistar rats, although some important differences also exist (Table 1-8).

Measures of acute inhalation neurotoxicity, namely $EC_{50}$ values for decreases in rotarod performance (4,694 and 4,738 mg/m$^3$) and pain sensitivity (5,683 and 5,963 mg/m$^3$), were similar for 1,2,4-TMB and 1,3,5-TMB, respectively (Korsak and Rydzyński, 1996). However, the $EC_{50}$ values for both measures were lower following exposure to 1,2,3-TMB (3,779 and 4,172 mg/m$^3$, respectively). The observation that 1,2,3-TMB may be slightly more neurotoxic than 1,2,4-TMB or 1,3,5-TMB was also observed following acute oral and injection exposures. Although all three isomers were observed to result in altered EEG readings, stronger and more persistent effects followed a pattern of 1,2,3-TMB > 1,3,5-TMB > 1,2,4-TMB after oral exposures (Tomas et al., 1999a) and 1,2,3-TMB > 1,2,4-TMB > 1,3,5-TMB after i.p. injections (Tomas et al., 1999c). Acute exposure to both 1,2,4-TMB and 1,2,3-TMB affected performance in open field tests at similar exposure levels, whereas 1,3,5-TMB appeared to be slightly more potent, although the magnitude of the response across isomers suggests that this difference is negligible (Tomas et al., 1999b).

In short-term neurotoxicity studies, a qualitatively similar pattern of effects (inability to learn passive and/or active avoidance and decreased pain sensitivity following footshock challenge) indicating altered neurobehavioral function was observed for TMBs, although some quantitative differences were noted (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998; Gralewicz et al., 1997b). Exposure to any isomer resulted in statistically significant decreases in pain sensitivity following footshock challenge at the same concentration, although the magnitude of effect and consistency across studies was greater for 1,3,5-TMB and

1,2,4-TMB compared to 1,2,3-TMB (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998; Gralewicz et al., 1997b). 1,2,4-TMB and 1,3,5-TMB were also observed to increase activity in open field tests, whereas 1,2,3-TMB was observed to have no statistically significant effects (Lutz et al., 2010; Wiaderna et al., 2002, 1998; Gralewicz et al., 1997b). In contrast, increased locomotor activity elicited by amphetamine was amplified following exposure to 1,2,3-TMB, but not 1,2,4-TMB (Lutz et al., 2010). All three isomers elicited effects on cognitive function, as measured by learning decrements in two-way active avoidance or by decreased fear responses in a passive avoidance test paradigm (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998; Gralewicz et al., 1997b). 1,3,5-TMB was observed to be the most potent isomer in this regard, eliciting effects on both passive and active avoidance at ≥123 mg/m$^3$. 1,2,3-TMB and 1,2,4-TMB affected passive avoidance performance at ≥123 and ≥492 mg/m$^3$, respectively, and both 1,2,3-TMB and 1,2,4-TMB affected the ability to learn active avoidance at 492 mg/m$^3$. For all isomers, short-term exposure to 1,230 mg/m$^3$ TMB was nearly always less effective (or ineffective), as compared to lower TMB concentrations, at eliciting responses (i.e., responses were nonlinear).

Following subchronic exposure to either 1,2,4-TMB or 1,2,3-TMB, both isomers decreased pain sensitivity and decreased rotarod performance. With regard to decreased pain sensitivity, although 1,2,3-TMB was observed to decrease pain sensitivity at a lower concentration than 1,2,4-TMB, the magnitude of effect was similar between isomers at every concentration (Korsak and Rydzyński, 1996). For either isomer, effects on pain sensitivity appeared to be reversible at 1,230 mg/m$^3$ TMB; lower concentrations were not tested. 1,2,3-TMB was more potent than 1,2,4-TMB in reducing rotarod performance. Specifically, 1,2,3-TMB elicited effects at a lower concentration and caused a greater magnitude of effect at each concentration, as well as following a period of recovery (Korsak and Rydzyński, 1996).

In acute studies investigating respiratory irritative effects (i.e., decreased respiratory rate), the $RD_{50}$ for the three isomers were very similar, ranging from 2,553 to 2,844 mg/m$^3$ (Korsak et al., 1997). Similarities were also observed in 1,2,4-TMB- and 1,3,5-TMB-induced developmental and maternal effects (Saillenfait et al., 2005). Male fetal weights were significantly reduced in animals exposed gestationally to 2,952 mg/m$^3$ 1,2,4-TMB (5% decrease) or 1,3,5-TMB (7% decrease). 1,2,4-TMB also significantly decreased female fetal weights by approximately 5% in animals exposed to the same concentration. Although 1,3,5-TMB significantly reduced female fetal weights by 13% in animals exposed to 5,904 mg/m$^3$, female fetal weights were decreased at 2,952 mg/m$^3$ to a similar degree (6%) as animals exposed to the same concentration of 1,2,4-TMB. Maternal toxicity, measured as decreased maternal weight gain, was observed in animals exposed to 2,952 mg/m$^3$ 1,2,4-TMB or 1,3,5-TMB. However, 1,3,5-TMB exposure resulted in a 75% reduction of maternal weight gain compared to controls, whereas 1,2,4-TMB exposure reduced maternal weight gain by 50%.

Lastly, 1,2,3-TMB, 1,2,4-TMB, and 1,3,5-TMB were observed to elicit hematological toxicity in exposed animals. Although all three isomers were observed to qualitatively affect similar hematological parameters, the direction and magnitude of effect often differed between isomers. RBCs were significantly decreased in male rats exposed to 1,230 mg/m$^3$ 1,2,3-TMB (23% decrease) or 1,2,4-TMB (15% decrease) (Korsak et al., 2000a, b). Reticulocyte numbers were also altered in rats following exposure to these isomers, although 1,2,4-TMB was observed to significantly decrease reticulocytes in male rats at 1,230 mg/m$^3$ (71% decrease), while exposure to 1,2,3-TMB increased reticulocytes in male rats at 1,230 mg/m$^3$ (61% increase) and female rats at 123 and 492 mg/m$^3$ (77 and 100% increases, respectively). 1,2,3-TMB and 1,2,4-TMB also altered the numbers of WBCs in exposed animals following subchronic exposures. In male rats exposed to 1,230 mg/m$^3$ 1,2,4-TMB, WBC numbers were significantly increased by 80%. Exposure to 1,230 mg/m$^3$ 1,2,3-TMB also increased lymphocyte numbers by 11 and 15% in male and female rats, respectively. Exposure to 1,230 mg/m$^3$ 1,2,3-TMB decreased segmented neutrophils by 29% in male rats, whereas exposure to 492 and 1,230 mg/m$^3$ decreased neutrophil numbers in female rats by 29 and 48%, respectively. Acute exposure (6 hours) to 1,500–6,000 mg/m$^3$ 1,3,5-TMB was also reported to result in increased numbers of segmented neutrophils that persisted for up to 28 days post-exposure (Wiglusz et al., 1975b).

In addition to similarities in observed toxicities among the individual TMB isomers, there are some similarities observed when considering the C9 aromatic fraction studies as well. For example, Clark et al. (1989) observed slight alterations to some hematological and clinical chemistry parameters that are similar to effects observed after exposure to 1,2,4-TMB and 1,2,3-TMB via inhalation. For example, in all studies, effects on the number of total WBCs, or specific types of lymphocytes, are observed. These alterations are possibly related to the respiratory irritative and/or inflammatory effects observed in the respiratory system following exposure. The effects observed in Clark et al. (1989) occur at similar concentrations of TMB isomers (approximately 800 mg/m$^3$) as in the individual TMB studies (492–1,230 mg/m$^3$). Similarly, Mckee et al. (1990) also observed developmental toxicity following exposure to the C9 fraction in the form of decreased fetal weight, similar to effects observed following exposure to 1,2,4-TMB and 1,3,5-TMB (Saillenfait et al., 2005), although Clark et al. (1989) additionally observed increases in fetal death and increased incidences of some malformations (e.g., cleft palate). However, some of the C9 fraction studies are also discordant with the larger TMB database. For example, Schreiner et al. (1989) did not observe any increase in SCE following in vitro assays, whereas Janik-Spiechowicz et al. (1998) observed that all three TMB isomers induced SCE when mice were exposed via i.p. injection. The starkest difference in toxicity between TMB isomers and the C9 fraction regards neurotoxicity. There is strong and consistent evidence that exposure to individual isomers of TMB causes neurotoxicity following short-term and subchronic exposures (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998; Gralewicz et al., 1997b; Korsak and Rydzyński, 1996), whereas subchronic exposure to the C9 fraction does not

appear to result in similar effects (Douglas et al., 1993). There are a number of possibilities for the observed differences in toxicity between TMB isomers and the C9 fraction. The specific test compound used in the C9 fraction was a complex aromatic hydrocarbon mixture reported to contain between 45 and 55% TMB isomers, with the remaining mixture primarily consisting of ethyltoluene isomers. However, the precise test agent to which animals were exposed was likely variable depending on the conditions under which they were generated across the different C9 fraction studies. Tertiary constituents (xylene, n-propyl- and isopropylbenzene, and unspecified C10 aromatic hydrocarbons) comprised as much as 16% of the test compound. Although a conclusion of sufficient toxicokinetic and toxicological similarity is used in the Toxicological Review to support the adoption of consistent, cross-isomer reference values, such a conclusion has not been tested for the other constituents of the C9 mixture. For some constituents (i.e., the C10 compounds), such a comparison is not possible as they were not specifically identified in the compositional analysis. Complex toxicokinetic interactions between different constituents of the C9 fraction are also possible that would result in altered distribution or metabolism of the individual constituents. For example, some components of the C9 fraction may have induced metabolic enzymes that cleared TMB isomers more rapidly, or may have competed for distributional pathways into target organs (i.e., preferentially distributed to the brain over TMB isomers). Some evidence does exist that co-exposures of rats to 1,3,5-TMB with the solvent ethyl acetate decreases the absorption of 1,3,5-TMB into the blood compared to rats only exposed 1,3,5-TMB (Freundt et al., 1989). It is possible that similar effects on TMB isomer uptake and/or absorption are mediated by the other constituents or impurities contained in the C9 fraction mixture.

Specifically considering decreased pain sensitivity, the difference in observed effects between TMB isomers and the C9 fraction may be due to study design differences rather than a fundamental difference in effects (see Section 1.2.1). Briefly, the failure of Douglas et al. (1993) to observe decreases in pain sensitivity may be due to when the endpoint was examined (24 hours post-exposure). This would be consistent with the findings of Korsak and Rydzyński (1996), where no effect was observed 2 weeks post-exposure, and short-term studies (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998; Gralewicz et al., 1997b), where no statistically significant effect was observed 50 days post-exposure. However, the observation of a latent effect at 51 days post-exposure (1 day post-environmental challenge) demonstrates that a persistent alteration of the nervous system of rats exposed to TMB isomers exists. Therefore, the apparent difference between the Douglas et al. (1993) C9 study and the TMB studies is potentially not a difference per se, but one piece of possibly confirmatory evidence when describing the entire time-course of TMB-induced decreases in pain sensitivity.

A summary of these comparisons across individual TMB isomers is presented in Table 1-8.

**Table 1-8. Similarities between 1,2,3-TMB, 1,2,4-TMB, and 1,3,5-TMB regarding observed inhalation and oral toxicity**

| Health outcome measure | Exposure duration | TMB isomer potency |
|---|---|---|
| Pain sensitivity | Acute | 1,2,3-TMB > 1,2,4-TMB ≈ 1,3,5-TMB |
| | Subchronic | 1,2,4-TMB ≈ 1,2,3-TMB |
| Pain sensitivity following footshock challenge | Short-term | 1,2,4-TMB ≈ 1,3,5-TMB > 1,2,3-TMB |
| Neuromuscular function | Acute | 1,2,3-TMB > 1,2,4-TMB ≈ 1,3,5-TMB |
| | Subchronic | 1,2,3-TMB > 1,2,4-TMB |
| Motor function/anxiety | Short-term | 1,2,4-TMB ≈ 1,3,5-TMB >> 1,2,3-TMB |
| Sensitization | Short-term | 1,2,3-TMB > 1,2,4-TMB |
| Cognitive function | Short-term | 1,3,5-TMB > 1,2,4-TMB ≈ 1,2,3-TMB |
| Electrocortical activity | Acute | 1,2,3-TMB >> 1,3,5-TMB > 1,2,4-TMB |
| Respiratory effects | Acute | 1,2,4-TMB ≈ 1,3,5-TMB ≈ 1,2,3-TMB |
| Developmental effects | Gestational | 1,2,4-TMB = 1,3,5-TMB |
| Hematological effects | Subchronic | 1,2,4-TMB ≈ 1,2,3-TMB |

## 1.3. SUMMARY AND EVALUATION

### 1.3.1. Weight of Evidence for Effects Other than Cancer

TMB isomers have been observed to exhibit many similarities in toxicokinetics in humans and animals across isomers. All three isomers have similar physiochemical properties and readily absorb into the bloodstream following inhalation exposures. Net respiratory uptake was similar across all three isomers in humans, and was similar in rats and humans for 1,2,4-TMB. The distributional pattern was similar across isomers in exposed rats, with the liver, lung, and kidneys identified as targets. 1,2,4-TMB was observed to distribute heavily to the brain, and while there was no distributional information for 1,2,3-TMB or 1,3,5-TMB, similarities in brain:air partition coefficients strongly suggest that distributional patterns would be similar across isomers. Brain:air partition coefficients are also similar in humans, suggesting that no appreciable difference in nervous system distributions patterns should be expected. All TMB isomers primarily metabolize to benzoic and hippuric acid metabolites, although the proportion of total metabolites that these individual metabolites comprised differed slightly across enzymes. Other metabolites included phenols, mercapturic acids, and glucuronides or sulphuric acid conjugates. The half-lives of elimination are multi-phasic for all isomers, with similar half-lives for the first three phases for all three isomers. However, the half-life for the last phase of elimination was much longer for 1,2,4-TMB compared to 1,3,5-TMB. Overall, these data support the conclusion that the TMB isomers are very similar to one another regarding their toxicokinetic characteristics.

In both humans and animals, inhalation exposure to TMBs has been shown to result in toxicity in multiple systems, including the nervous, respiratory, and hematological systems. In addition, developmental toxicity has been observed in animals exposed to either 1,2,4-TMB or 1,3,5-TMB. Generally, the information regarding inhalation toxicity in humans is limited for a number of reasons, including that the majority of human studies involved exposure to complex VOC mixtures containing several TMB isomers and other VOCs, and not the individual isomers themselves. Therefore, the observed health effects cannot be attributed to specific TMB isomers. However, these studies observe effects in exposed human populations that are generally analogous to effects observed in animal toxicity studies, and provide qualitative, supportive evidence for hazard identification. Currently, no human studies exist that investigate the oral toxicity of any TMB isomer.

The most strongly and widely supported manifestation of toxicity in humans and animals following inhalation exposure to 1,2,3-TMB, 1,2,4-TMB, or 1,3,5-TMB is neurotoxicity (see Summary in Section 1.2.1). In humans exposed to TMB-containing VOC mixtures, a multitude of effects, including neuropsychological effects (El Hamid Hassan et al., 2013; Chen et al., 1999), deficits in short-term memory and reduced motor speed/coordination (Lee et al., 2005; Ruijten et al., 1994), abnormal fatigue (Norseth et al., 1991), dysfunction of the inner ear/vertigo (Juárez-Pérez et al., 2014; Fuente et al., 2013; Sulkowski et al., 2002), visual dysfunction (Gong et al., 2003; Pratt et al., 2000), and nervousness, anxiety, and/or vertigo (Bättig et al. (1956), as reviewed by MOE (2006) and Bättig et al. (1958)) have been observed. None of the available human studies have addressed the potential for latent neurological effects, and no studies examined the potential for neurological effects in sensitive populations. Although the reported human symptoms do not directly parallel the animal data, exposure of male Wistar rats to the TMB isomers has been shown to consistently result in a multitude of neurotoxic effects, including decreased pain sensitivity, impaired neuromuscular function and coordination, altered cognitive function, decreased anxiety and/or increased motor function in open field tests, and neurophysiological effects (e.g., decreased electrocortical activity) across multiple concentrations and durations (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998; Gralewicz et al., 1997b; Gralewicz et al., 1997a; Korsak and Rydzyński, 1996; Korsak et al., 1995). Some differing evidence does exist regarding the potential for neurotoxicity following exposure to C9 mixtures: exposure to the C9 fraction (resulting in concentrations of total TMB isomers much higher than those used in individual TMB isomer studies) failed to elicit a similar pattern of neurotoxic responses to those observed in isomer-specific studies. However, this may be due more to the particular study designs used rather than true differences in toxicity between the total C9 fraction and its individual constituents (see Sections 1.2.1 and 1.2.7).

The effects observed in animals in the TMB isomer neurotoxicity studies are recognized in EPA's *Guidelines for Neurotoxicity Risk Assessment* (U.S. EPA, 1998) as possible indicators of neurotoxicity. The effects include concentration-dependent decrements in pain sensitivity in hot

plate tests and neuromuscular function in rotarod tests following subchronic exposure. Although effects on pain sensitivity appeared to be reversible at the highest concentration (i.e., 1,230 mg/m$^3$), a multitude of other adverse endpoints indicates that neurotoxicity is not reversible, including latent effects of short-term TMB exposure on potentially more sensitive indicators of pain sensitivity (i.e., hot plate tests following an environmental [footshock] challenge), as well as persistent neuromuscular dysfunction in rotarod tests following subchronic exposure, and reproducible learning decrements in passive and active avoidance experiments, altered EEG patterns, and increased locomotor activity in open field tests weeks to months after short-term exposure. The data from short-term exposure studies indicated a consistent nonlinearity in many of the TMB-elicited responses observed sometime after exposures had ended (e.g., 1,230 mg/m$^3$ was nearly always substantially less effective than 123 or 492 mg/m$^3$), which may be related to the specific mode(s) of action for these latent effects, which remains unknown. The neurotoxic effects are biologically plausible and analogous to effects that could occur in humans. Thus, the evidence for TMBs identifies neurotoxicity as a toxicity hazard based on consistency and coherency of effect across multiple studies and durations of exposure.

Three acute oral studies (Tomas et al., 1999a; Tomas et al., 1999b; Tomas et al., 1999c) exist that reported similar effects as observed in the available inhalation neurotoxicity studies (i.e., increased locomotor activity and altered brain wave activity). However, these studies are limited with regard to their duration (i.e., acute) and nature of endpoints investigated, and as such, no weight-of-evidence determination can be made regarding the oral toxicity of the TMB isomers.

In addition to neurotoxicity, both respiratory and hematological toxicity have been observed in human populations and animals exposed to TMBs, or to mixtures containing the three isomers. In humans, occupational and residential exposures to VOC mixtures containing TMB isomers have resulted in number of effects characterized as respiratory toxicity, including asthmatic bronchitis (Bättig et al. (1956), as reviewed in MOE (2006) and Bättig et al. (1958)), asthma (Billionnet et al., 2011), or laryngeal/pharyngeal irritation (Norseth et al., 1991). Additionally, workers exposed to a VOC mixture containing 1,2,4-TMB and 1,3,5-TMB, and possibly 1,2,3-TMB, were reported to exhibit hematological effects including alterations in clotting time and anemia (Bättig et al. (1956), as reviewed in MOE (2006) and Bättig et al. (1958)). Again, as workers were exposed to complex VOC mixtures containing TMB isomers, the observed health effects cannot be attributed to any single TMB isomer.

The observation of respiratory irritation and inflammation in Wistar rats and BALB/C mice following exposure to 1,2,4-TMB was consistent across multiple concentrations, and subchronic and acute exposure durations (Korsak et al., 2000a; Korsak et al., 1997; Korsak et al., 1995). Respiratory toxicity was also observed in multiple studies involving exposure to 1,2,3-TMB (Korsak et al., 2000b; Korsak et al., 1995). Some inflammatory lesions similar to those observed in individual isomer studies (pulmonary macrophage infiltration and alveolar wall thickening) were also observed following exposure to the C9 fraction (Clark et al., 1989). Although the reported

symptoms in humans (laryngeal and/or pharyngeal irritation, asthmatic bronchitis, and asthma) do not directly parallel the effects observed in animal studies, the observation of irritative and/or inflammatory responses in multiple species (including humans) demonstrates a consistency in TMB-induced respiratory toxicity. Additionally, multiple measures of hematological toxicity have been observed in rats subchronically exposed to 1,2,4-TMB or 1,2,3-TMB, including decreased RBCs, increased WBCs, decreased clotting time, and decreased reticulocytes (1,2,4-TMB) and decreased RBCs, decreased segmented neutrophils, increased lymphocytes, and increased reticulocytes (1,2,3-TMB) (Korsak et al., 2000a, b). At least two of these effects, decreased RBCs and decreased clotting time, are roughly analogous to the hematological effects (alterations in clotting and anemia) observed in occupationally exposed humans, thereby demonstrating a consistency and coherency of effect across species. Some hematological and clinical chemistry effects (decreased osmotic fragility, decreased PCV, decreased RBCs, increased AP and AST) were also observed following exposure to the C9 fraction (Carrillo et al., 2014; Clark et al., 1989). Therefore, the respiratory and hematological effects observed in animals are biologically plausible and analogous to effects that could occur in exposed human populations. The available weight of evidence for 1,2,4-TMB and 1,2,3-TMB identified respiratory and hematological toxicity as a hazard.

Currently, no human studies exist that investigate the reproductive or developmental toxicity of 1,2,3-TMB, 1,2,4-TMB, or 1,3,5-TMB. However, one animal study (Saillenfait et al., 2005) observed effects on fetal body weights and maternal body weight gains due to exposure during gestation to 1,2,4-TMB or 1,3,5-TMB. Exposure to the C9 fraction also induced developmental toxicity: mice exposed to the C9 fraction experienced decreased fetal survival and increased malformations (Mckee et al., 1990). In rats exposed to the C9 fraction, decreased male fertility was observed and some suggestions of intergenerational effects on growth (exacerbated body weight decrements in later generations) were observed in a multi-generational reproductive toxicity study (Mckee et al., 1990); no individual isomer reproductive toxicity studies currently exist. Although the weight of evidence regarding developmental toxicity is not as strong compared to other measures of toxicity in the TMB database, these effects observed in animals are considered biologically plausible and potentially analogous to effects that could occur in humans. The available evidence for 1,2,4-TMB and 1,3,5-TMB identifies maternal and developmental toxicity as a hazard.

Although no compelling TMB isomer-specific data exist by which to draw firm conclusions on possible modes of action, data from other lines of evidence (related compounds, mixtures) can be used to tentatively identify possible modes of action. One possible overarching mode of action for effects in multiple systems is oxidative stress. 1,2,4-TMB has been observed to result in increased reproductive burst in neutrophils, which may possibly be relevant to brain injury, as microglia cells have a respiratory burst similar to neutrophils (Myhre et al., 2000). Additional evidence of an oxidative stress mode of action is the observation that rat neural synaptosomes exposed to 1,2,4-TMB produced a dose-dependent increase in ROS and reactive nitrogen species demonstrated by the formation of the fluorescence of 2'7'-dichlorofluorescein (Myhre and Fonnum,

2001) and the observation that toluene exposure increases the concentration of oxygen radicals in the brain (Tormoehlen et al., 2014). These observations of ROS production in rat synaptosomes may potentially explain the observed TMB-induced neurotoxicity in acute, short-term, and subchronic inhalation studies. Although pulmonary ROS generation has not been observed following in vivo or in vitro TMB exposures, related compound such as benzene and toluene have been shown to induce oxidative stress in cultured lung cells (Mögel et al., 2011). This suggests that TMB-induced irritative and inflammatory responses may also be due to an oxidative stress mode of action.

Although the TMB toxicity database is relatively complete in some areas (e.g., neurotoxicity testing in adult animals), there do exist some important limitations and data gaps in the database. In general, the available human data are not specific to TMBs, and provide only limited qualitative support. Most notably, there are no chronic studies available that investigate health effects in human populations exposed to TMB isomers individually via inhalation. While there are a number of occupational epidemiologic studies in the database, the workers in these studies were exposed to complex solvent mixtures that contained not only TMB isomers, but also other aliphatic and aromatic compounds. Therefore, as stated above, these studies can provide qualitative support for hazard identification as they report effects that are generally analogous to effects observed in animal toxicity studies. Currently, no human studies exist that investigate the oral toxicity of any TMB isomer.

There are also a number of potential limitations in the animal inhalation and oral toxicity database for TMBs, including the lack of a chronic study for any individual TMB isomer, the lack of a subchronic neurotoxicity test for 1,3,5-TMB, and the fact that all of the available short-term or subchronic inhalation animal studies were conducted by the same research group: The Nofer Institute of Occupational Medicine, Lodz Poland. Although no chronic studies exist for individual TMB isomers, a chronic study investigating the effects of exposure to the C9 fraction is available that reports effects similar to those observed in the subchronic inhalation studies of individual TMB isomers (i.e., effects on respiratory, hematological, and clinical chemistry effects), but failed to assess the potential for the most prominent health effect caused by the individual isomers (i.e., neurotoxicity). Other data gaps in the TMB toxicity database include the lack of a developmental neurotoxicity study or a multi-generational reproductive toxicity study for any individual TMB isomer. There is a multi-generational reproductive/developmental toxicity study for the C9 fraction (Mckee et al., 1990) that reported possible reproductive toxicity in rats (increased time to mating, decreased male fertility) and a suggestive intergenerational effect on pup weight in rats. While a study on the developmental neurotoxicity of Aromatol reported no observed effects (Lehotzky et al., 1985), a number of review articles on related compounds reported that gestational exposure to toluene (humans) or toluene or xylene (animals) results in developmental neurotoxicity (delayed righting reflexes, decrements in spatial learning, and altered

behavioral responses) (Grandjean and Landrigan, 2014; Hannigan and Bowen, 2010; Win-Shwe and Fujimaki, 2010; Bowen and Hannigan, 2006; Grandjean and Landrigan, 2006; Ritchie et al., 2001).

While evidence from related compounds can identify possible data gaps in the TMB database, this additional evidence stream can also provide confirmatory support for health effects that are observed in the TMB database. Multiple reviews of the alkylbenzene literature report that occupationally exposed humans suffer neurotoxic effects (e.g., color discrimination, neuropsychological symptoms, decreased reaction times, impaired postural equilibrium, etc.) that are functionally similar to those observed in adults exposed to mixtures containing TMB isomers (Gobba and Cavalleri, 2003; Ritchie et al., 2001; Costa, 1996), while alkylbenzene exposure is observed to result in ototoxicity, altered operant conditioning, and increased locomotor activity in adult animals (Ritchie et al., 2001). Information from the C9 fraction or related compounds and/or mixtures support the observation of developmental toxicity following TMB exposure. Exposure to the C9 fraction resulted in substantial developmental toxicity in mice, including increased fetal death, decreased fetal weights, and increases in particular malformations and developmental variations (i.e., cleft palate and unossified sternebrae) (Mckee et al., 1990). Developmental toxicity (fetal death, skeletal malformations, spontaneous abortions, and decreased fetal weights) was observed in rats, mice, and rabbits exposed to benzene, toluene, xylene, ethylbenzene, or Aromatol (Ungvary and Tatrai, 1985). Limited evidence also exists that demonstrates associations between alkylbenzenes and increased risk of asthma and decreased lung function and between benzene and decreased hemoglobin, hematocrit, and RBC counts (Bolden et al., 2015).

In summary, the overall evidence for TMB isomers strongly supports a determination that exposure to TMBs results in adverse health effects in numerous systems, including the nervous, respiratory, and hematological systems. This determination is based on consistency and coherency of effects in humans and animals, biological plausibility, and observed exposure-response relationships in animals. The evidence for reproductive and developmental toxicity is weaker than the aforementioned effects, but evidence in animals indicates that exposure to TMBs does elicit some measures of reproductive and developmental toxicity. Given the observation of similar types of toxicity endpoints in animals exposed to related alkylbenzenes, there is increased confidence that observed TMB isomer hazards have been adequately identified, even when considering that the TMB database is lacking in some areas (e.g., no chronic studies, limited to no information on mode of action). The health effects identified as hazards for TMBs will be considered further for their utility in quantitatively deriving reference values for the individual TMB isomers; these considerations and derivations are included in Section 2: Dose-Response Analysis.

### 1.3.2. Weight of Evidence for Carcinogenicity

Under the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005), there is "inadequate information to assess carcinogenic potential" of TMBs. This characterization is based on the fact that there is no information regarding the carcinogenicity of TMBs in humans and that the only animal study available on the carcinogenicity of 1,2,4-TMB observed no statistically significant

carcinogenic effects. No studies regarding the carcinogenicity of 1,2,3-TMB or 1,3,5-TMB were identified in the available scientific literature.

In the animal carcinogenicity study (Maltoni et al., 1997), involving exposure to 1,2,4-TMB by gavage, an increased incidence of total malignant tumors in both sexes and head cancers (including the rare neuroethesioepithelioma in one male and two females) was observed in exposed rats; no statistical analyses were reported.

In the only study investigating the genotoxicity of TMB isomers, Janik-Spiechowicz et al. (1998) observed negative results in in vitro genotoxicity assays (i.e., Ames mutation assay in Salmonella) involving 1,2,4-TMB and 1,3,5-TMB. However, 1,2,3-TMB was observed to induce gene mutations in all *S. typhimurium* strains tested. All three isomers failed to induce micronuclei in mouse bone marrow cells. Janik-Spiechowicz et al. (1998) observed an increased incidence of SCE in mice exposed to all three TMB isomers (individually); however, this observation does not provide a specific indication of mutagenic potential. Given the findings regarding the in vitro genotoxicity of the TMB isomers, and the fact that increased frequency of SCEs does not provide specific indication of mutagenic potential, the evidence is inadequate to conclude that any TMB isomer is genotoxic.

### 1.3.3. Susceptible Populations and Lifestages

Although there are no TMB-specific data that would allow for the identification of susceptible populations and lifestages, the reduced metabolic and elimination capacities in children relative to adults may be a source of susceptibility (Ginsberg et al., 2004). TMB isomers are metabolized following inhalation and oral exposure via side-chain oxidation to form alcohols and aromatic carboxylic/mercapturic acids or by hydroxylation to form phenols, which are then conjugated with glucuronic acid, glycine, or sulfates for urinary excretion. The activities of multiple CYP450 mono-oxygenase isozymes have been shown to be reduced in children up to 1 year of age compared to adult activities (Ginsberg et al., 2004). Currently, it is not known which CYP450 isozyme is responsible for TMB metabolism, although CYP2E1 is the major CYP450 isozyme responsible for the metabolism of low molecular weight VOCs (Nong et al., 2006). If that is also the case for TMB isomers, the lower activity of the CYP2E1 isozyme in children up to 1 year of age (27–47% compared to Ginsberg et al., 2004) could result in newborns and young infants experiencing higher and more persistent blood concentrations of the un-metabolized TMB isomers. This is important as it is currently assumed that the parent TMB isomers are the toxic moieties. When modeling inter-child differences in pharmacokinetics of toluene, a clear difference was observed in the intrinsic clearance of toluene based on CYP2E1 content and liver weight in young children <11 years old (0.03–1.05 L/minute) versus adolescents and adults (3.49–3.52 L/minute) (Nong et al., 2006). Incorporating these differences into a PBPK model and estimating venous blood concentrations of toluene, neonates (<1 month old) had maximum ($C_{max}$) toluene blood levels ranging from 0.21 to 0.70 µg/mL compared to 0.110.29 µg/mL in adults and other children. The difference was even larger when comparing the toluene blood levels of low-metabolizing neonates,

who had blood toluene levels of 0.51–0.71 µg/mL. Similar effects were obtained when investigating cumulative doses (area under the curve, AUC). In order to estimate a chemical-specific intra-human pharmacokinetic uncertainty factor ($UF_{H\text{-}PK}$) for toluene, the mean adult AUC was compared to the 95$^{th}$ percentile AUC for low metabolizing neonates resulting in an estimated $UF_{H\text{-}PK}$ of 3.9, slightly higher than the EPA value of 3.16 (i.e., $\sqrt{10}$); a value of 2.5 was estimated for high metabolizing infants, and no other age-group returned values >1.6.

Ginsberg et al. (2004) also demonstrates that the rate of glucuronidation and sulfation is decreased in neonates and children up to 2 months of age (34–47% compared to adults), resulting in possible prolonged exposure to the metabolites of TMB isomers. If TMB metabolites also confer some toxicity to exposed children, decreased glucuronidation in young children may increase their susceptibility to the various hazards identified for TMB isomers. Reduced renal clearance in children may be another important source of potential susceptibility. TMB isomers and their metabolites are excreted in the urine of exposed laboratory animals and occupationally exposed humans. Data indicating reduced renal clearance for infants up to 2 months of age (Ginsberg et al., 2004) may suggest a potential to affect TMB excretion, thus possibly prolonging its toxic effects. Additionally, those with pre-existing respiratory diseases (e.g., asthma) may be more sensitive to the respiratory irritative and inflammatory effects of TMB isomers. Genetic polymorphisms that alter the expression or activity of enzymes that mediate the normal, homeostatic metabolism of neurotransmitters may also increase the susceptibility of carriers of those polymorphisms to environmental neurotoxicants (Costa, 1996).

Some research into toluene provides evidence that the postnatal period may be a sensitive time point for VOC-induced neurotoxicity. During the early neonatal period, a number of neurodevelopmental processes, including synaptogenesis, gliogenesis, and myelination, are ongoing (Win-Shwe and Fujimaki, 2010). Exposure of mouse pups to 5 ppm toluene via inhalation on PNDs 8–12 resulted in the upregulation of multiple neuroimmune markers in the hippocampus (Win-Shwe et al., 2012). Additionally, inhalation exposure to the same level of toluene (5 ppm) on PNDs 8–12 was shown to upregulate the expression of signal transduction receptors in the hippocampus that are essential to spatial learning and memory (Win-Shwe et al., 2010), which correlated with the results of neurobehavioral testing of pups on PND 49, including treatment-related decrements in spatial learning using a water maze. As these effects were not observed in animals exposed on PNDs 14–18, these data identify PNDs 8–12 as a potential sensitive window of susceptibility for neurodevelopmental effects. Consistent with this, decreases in spatial learning and memory were observed in postnatally exposed animals at 5 ppm, whereas similar effects were only observed in animals exposed as adults at 80 ppm (von Euler et al., 1993). Exposure to toluene during early life has also been observed to perturb pathways related to pain sensitivity: neonatal rats exposed to 500 mg/kg toluene i.p. on PNDs 4–9 had reduced sensitivity to nicotine-induced antinociception (meaning that toluene exposure prevented nicotine-induced decreases in pain sensitivity) (Chan et al., 2008). Together, these data indicate that infants may represent a

particularly susceptible lifestage for the effects of exposure to compounds related to TMB isomers. Other reviews of the VOC literature further indicate that toluene exposure during gestation or early life can result in developmental neurotoxicity (Grandjean and Landrigan, 2014; Hannigan and Bowen, 2010; Win-Shwe and Fujimaki, 2010; Bowen and Hannigan, 2006; Grandjean and Landrigan, 2006; Ritchie et al., 2001), although often due to maternal inhalant abuse in human populations or resulting from animals studies utilizing study designs meant to approximate inhalant abuse patterns (i.e., high doses, intermittent exposures). It is unclear how these paradigms would relate to constant, low-level environmental exposures.

Therefore, although there exist no TMB-specific data with which to estimate early-life vulnerability, some data gleaned from the related compound, toluene, do provide some suggestive evidence that periods in early life represent periods of susceptibility to solvent exposure. Therefore, it can be reasonably assumed that exposures in early life to individual TMB isomers are of particular concern.

# 2. DOSE-RESPONSE ANALYSIS

This Dose-Response Analysis section details the derivation of chronic reference concentration (RfC) and reference dose (RfD) values for all three trimethylbenzene (TMB) isomers for specific organ-systems (nervous, respiratory, and hematological systems as well as pregnant animals and the developing fetus), as well as an overall chronic RfC and RfD value for TMB isomers as a group. This last derivation stems from the conclusions of the Hazard Identification section in which the similarity in toxicological profiles between TMB isomers was extensively reported. This conclusion supports decisions in this section to adopt RfC or RfD values for one isomer as the RfC or RfD value for another isomer when data are lacking for the second isomer. Regarding the RfD, only one isomer-specific oral study was available that could support the derivation of a chronic RfD. This study reported low levels of toxicity in the hematological system and did not investigate neurotoxicity endpoints. As neurotoxicity was identified as the critical effect domain in the Hazard Identification section, a route-to-route extrapolation was performed to extrapolate inhalation data to oral exposures to support the derivation of a neurotoxicity-based RfD. This neurotoxicity RfD was then compared to the hematological RfD for final selection of the overall TMB RfD. Lastly, subchronic RfC and RfD values were derived for less-than-lifetime exposures.

## 2.1. INHALATION REFERENCE CONCENTRATION FOR EFFECTS OTHER THAN CANCER FOR TMBs

The RfC (expressed in units of $mg/m^3$) is defined as an estimate (with uncertainty spanning perhaps an order of magnitude) of a continuous inhalation exposure to 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 no- or lowest-observed-adverse-effect level (NOAEL or LOAEL), or the 95% lower bound on the benchmark concentration (BMCL), with uncertainty factors (UFs) generally applied to reflect limitations of the data used.

### 2.1.1. Identification of Studies and Effects for Dose-Response Analysis and Derivation of Reference Concentrations for TMBs

Multiple systems have been identified as targets of inhaled TMB isomers in humans and experimental animals, and effects in these systems have been identified as hazards following inhalation exposure to these isomers. In humans and experimental animal models, the nervous, respiratory, and hematological systems have been identified as targets following exposure to 1,2,4-TMB, 1,2,3-TMB, and 1,3,5-TMB. Additionally, pregnant animals and the developing fetus have been identified as targets of inhaled 1,2,4-TMB and 1,3,5-TMB in experimental animal models.

No human studies exist to characterize the potential toxicity for pregnant women and fetuses following inhalation exposure to TMB isomers, and no toxicological study exists to characterize this hazard in animals exposed to 1,2,3-TMB.

The selection of studies and general procedures for dose-response analysis are discussed in Sections 7 and 8 of the Preamble. Human data are preferred over animal data for deriving reference values when possible because the use of human data is more relevant in the assessment of human health and avoids the uncertainty associated with interspecies extrapolation introduced when animal data serve as the basis for the reference value. In this case, while literature exists on the effects of 1,2,4-TMB, 1,2,3-TMB, and 1,3,5-TMB exposure in humans, including neurological, respiratory, and hematological toxicities, no human studies are available that would allow for dose-response analysis. The human studies evaluated TMB exposures occurring as complex solvents or volatile organic carbon (VOC) mixtures, and this confounding along with other uncertainties including high imprecision in effect measures due to low statistical power, lack of quantitative exposure assessment, and lack of control for co-exposures, limit their utility in derivation of quantitative human health toxicity values. However, these studies provide supportive evidence for the neurological, respiratory, and hematological toxicity of TMB isomers in humans and indicate a coherency of effects in both humans and laboratory animals.

The available animal toxicity studies evaluating health effects following exposure to the C9 fraction (Douglas et al., 1993; Mckee et al., 1990; Clark et al., 1989) were similarly not considered for the derivation of RfC values given that approximately half of the mixture was comprised of chemicals other than the three TMB isomers. Significant uncertainty exists in these studies regarding whether or not co-exposure with other alkylbenzenes altered the distribution or metabolism of TMB isomers, and whether antagonistic interactions between constituents influenced the toxicodynamics of individual components of the mixture. As such, given this lack of knowledge, an assumption that the C9 fraction would be an adequate surrogate for individual TMB isomers is not justified. Therefore, given this uncertainty, and the existence of multiple suitable individual TMB isomer studies, the C9 fraction studies were excluded from consideration for the purpose of identifying a principal study.

Several studies investigating 1,2,4-TMB-, 1,2,3-TMB-, and 1,3,5-TMB-induced effects in experimental animal models were identified in the literature. No chronic studies were available, although acute, short-term, subchronic, and developmental toxicity studies were identified. 1,2,4-TMB- and 1,2,3-TMB-induced toxicity was observed across several systems in four subchronic studies by Korsak and colleagues (Korsak et al., 2000a, b; Korsak et al., 1997; Korsak and Rydzyński, 1996). One developmental toxicity study investigating maternal and fetal toxicity following exposure to either 1,2,4-TMB or 1,3,5-TMB was identified in the literature (Saillenfait et al., 2005). These studies were the only subchronic or developmental studies identified in the peer-reviewed literature. Data from these studies pertaining to the hazards observed in humans and animals identified in Chapter 1 (neurological, respiratory, and hematological toxicity) or in animals

only (maternal and developmental toxicity) were considered as critical effects for the purpose of determining the point of departure (POD) for derivation of the inhalation RfC for TMB isomers. In addition to effects following subchronic exposure, neurotoxicity was observed in both acute and short-term inhalation studies and respiratory toxicity was also observed in acute studies. However, the high concentrations used in acute studies and the short exposure durations employed in both acute and short-term studies limit their utility for the quantitation of chronic human health effects (e.g., several studies investigating relevant neurotoxicity endpoints (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998; Gralewicz et al., 1997b) only exposed animals for 28 days, and are therefore less suitable for derivation of chronic RfCs). Thus, the studies of subchronic exposure duration were preferred and the short-term and acute studies on these health endpoints were not selected. Nevertheless, as with the human mixture studies, these studies of shorter exposure durations do provide qualitative information regarding hazard identification, especially the observation of the consistency and coherency of these effects across the TMB database.

The four subchronic studies by Korsak and colleagues (Korsak et al., 2000a, b; Korsak et al., 1997; Korsak and Rydzyński, 1996), and the developmental toxicity study by Saillenfait et al. (2005), are adequate for dose-response analysis. All of these studies used rats as an appropriate laboratory animal species, and utilized appropriate sham-exposed controls. Animals were exposed to 1,2,4-TMB, 1,2,3-TMB, or 1,3,5-TMB reported as ≥97–99% pure (impurities not reported). These studies utilized an appropriate route (inhaled air) and duration (subchronic or gestational) of exposure. The subchronic and gestational studies used a reasonable range of appropriately-spaced exposure levels to facilitate dose-response analysis. An appropriate latency between exposure and development of toxicological outcomes was used, and the persistence of some outcomes (i.e., neurotoxicity and hematological effects) after termination of exposure was investigated. Adequate numbers of animals per exposure group were used, and appropriate statistical tests including pair-wise and trend analyses were performed. With regard to reporting of exposure methodologies, actual concentrations, as measured by gas chromatography, were reported to be within 10% of target concentrations (Saillenfait et al., 2005; Korsak et al., 2000a, b). This increases the confidence in the overall evaluation and adequacy of these studies. Although Korsak and Rydzyński (1996) and Korsak et al. (1997) do not report actual, measured concentrations, these studies use the same exposure methodology as Korsak et al. (2000a), suggesting that it is likely that the actual concentrations in these studies were also within 10% of target concentrations. Target and actual concentrations are presented in Table 2-1.

**Table 2-1. Target and actual exposure concentrations used in BMD modeling of 1,2,4-TMB, 1,2,3-TMB, and 1,3,5-TMB endpoints considered for the derivation of the RfC**

| Reference | Species/sex | Isomer | Target exposure concentration ($mg/m^3$) | Actual exposure concentration ($mg/m^3$)[a] |
|---|---|---|---|---|
| Korsak and Rydzyński (1996) | Rat, male | 1,2,4-TMB; 1,2,3-TMB | 123 | N/A |
| | | | 492 | N/A |
| | | | 1,230 | N/A |
| Korsak et al. (1997) | Rat, male | 1,2,4-TMB | 123 | N/A |
| | | | 492 | N/A |
| | | | 1,230 | N/A |
| Korsak et al. (2000a) | Rat, male and female | 1,2,4-TMB | 123 | 129 |
| | | | 492 | 492 |
| | | | 1,230 | 1,207 |
| Korsak et al. (2000b) | Rat, male and female | 1,2,3-TMB | 123 | 128 |
| | | | 492 | 523 |
| | | | 1,230 | 1,269 |
| Saillenfait et al. (2005) | Rat, female (pregnant dam); male and female (fetuses) | 1,2,4-TMB | 492 | 492 |
| | | | 1,476 | 1,471 |
| | | | 2,952 | 2,913 |
| | | | 4,428 | 4,408 |
| | | 1,3,5-TMB | 492 | 497 |
| | | | 1,476 | 1,471 |
| | | | 2,952 | 2,974 |
| | | | 5,904 | 5,874 |

[a]Actual exposure concentrations, when available, were used for dose inputs for benchmark dose (BMD) modeling.

These subchronic and developmental toxicity studies examined TMB-induced toxicity in multiple systems and neurological, respiratory, hematological, maternal, or developmental toxicity endpoints that demonstrated statistically significant pair-wise increases or decreases relative to control were considered for the derivation of the RfC for TMBs (Tables 2-2 and 2-3).

**Table 2-2. Endpoints observed in rats in the Korsak studies (Korsak et al., 2000a, b; Korsak et al., 1997; Korsak and Rydzyński, 1996) considered for the derivation of the RfC for TMBs**

| Endpoint | Isomer | Sex | Exposure concentration (mg/m³)[a] | | | |
|---|---|---|---|---|---|---|
| | | | 0 | 123 | 492 | 1,230 |
| **Neurological** | | | | | | |
| Decreased pain sensitivity (sec)[b] | 1,2,4-TMB | Male | 15.4 ± 5.8 (n = 9) | 18.2 ± 5.7 (n = 10) | 27.6 ± 3.2** (n = 9) | 30.1 ± 7.9** (n = 10) |
| | 1,2,3-TMB | Male | 9.7 ± 2.1 (n = 30) | 11.8 ± 3.8* (n = 20) | 16.3 ± 6.3c (n = 10) | 17.3 ± 3.4** (n = 10) |
| Decreased neuromuscular function (% failure)[d] | 1,2,4-TMB | Male | 0 (n =10) | 30 (n =10) | 40** (n =10) | 40** (n =10) |
| | 1,2,3-TMB | Male | 0 (n =10) | 40** (n =10) | 60** (n =10) | 70** (n =10) |
| **Hematological** | | | | | | |
| Decreased RBCs ($10^6$/mm³)[e] | 1,2,4-TMB | Male | 9.98 ± 1.68 (n = 10) | 9.84 ± 1.82 (n = 10) | 8.50 ± 1.11 (n = 10) | 7.70 + 1.38** (n = 10) |
| | 1,2,3-TMB | Male | 9.49 ± 2.03 (n = 10) | 10.2 ± 1.29 (n = 10) | 10.11 ± 1.27 (n = 10) | 8.05 ± 1.38* (n = 10) |
| Increased WBCs ($10^3$/mm³)[e] | 1,2,4-TMB | Male | 8.68 ± 2.89 (n = 10) | 8.92 ± 3.44 (n = 10) | 8.30 ± 1.84 (n = 10) | 15.89 ± 5.74** (n = 10) |
| Decreased segmented neutrophils (%)[f] | 1,2,3-TMB | Male | 24.8 ± 4.5 (n = 10) | 25.4 ± 5.8 (n = 10) | 20.7 ± 5.8 (n = 10) | 17.7 ± 8.3* (n = 10) |
| | | Female | 23.1 ± 6.1 (n = 10) | 19.7 ± 3.4 (n = 10) | 16.4 ± 4.2* (n = 10) | 11.9 ± 7.1** (n = 10) |
| Decreased reticulocytes (%)[e] | 1,2,4-TMB | Female | 3.5 ± 2.6 (n = 10) | 1.7 ± 2.0 (n = 10) | 1.8 ± 0.9 (n = 10) | 1.0 ± 0.6* (n = 10) |
| Increased reticulocytes (%)[f] | 1,2,3-TMB | Male | 2.8 ± 1.3 (n = 10) | 2.1 ± 1.7 (n = 10) | 3.8 ± 2.1 (n = 10) | 4.5 ± 1.8* (n = 10) |
| Decreased clotting time (sec)[d] | 1,2,4-TMB | Female | 30 ± 10 (n = 10) | 23 ± 4 (n = 10) | 19 ± 5** (n = 10) | 22 ± 7* (n =10) |
| **Respiratory** | | | | | | |
| Inflammatory lung lesions[e,f] | 1,2,4-TMB | Male | [h] (n = 10) | [h] (n = 10) | [h] (n = 10) | [h] (n = 10) |
| | 1,2,3-TMB | Female | [h] (n = 10) | [h] (n = 10) | [h] (n = 10) | [h] (n = 10) |
| Increased bronchoalveolar total cells ($10^6$/cm³)[g] | 1,2,4-TMB | Male | 1.93 ± 0.79 (n = 6) | 5.82 ± 1.32*** (n = 6) | 5.96 ± 2.80** (n = 7) | 4.45 ± 1.58* (n = 7) |

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

[a]Values are expressed as mean ± 1 standard deviation (SD). Korsak and Rydzyński (1996) does not explicitly state that the reported measures of variance in Table 1 of that reference are SDs. However, independent analysis conducted by EPA confirms that the reported measures of variance are SDs.

[b]Adapted from Korsak and Rydzyński (1996), measured as latency to paw-lick.

[c]Level of significance not reported in Table 1 from Korsak and Rydzyński (1996); however, the results of an ad-hoc t-test (performed by EPA) indicated significance at $p < 0.01$.

[d]Adapted from Korsak and Rydzyński (1996), measured as percent failure on the rotarod apparatus.

[e, f, g]Adapted from Korsak et al. (2000a), Korsak et al. (2000b), and Korsak et al. (1997), respectively.

[h]Incidences for individual exposure groups not reported; however, based on qualitative information reported in the study (i.e., that male or female rats exhibited a statistically significant increase in inflammatory lung lesions at 492 mg/m³), a NOAEL of 123 mg/m³ was identified for both isomers.

**Table 2-3. Endpoints observed in rats in the Saillenfait et al. (2005) study considered for the derivation of the RfC for TMBs**

| Endpoint | Isomer | Sex | Exposure concentration (mg/m³)[a] | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0 | 492 | 1,476 | 2,952 | 4,428 | 5,904 |
| **Maternal** | | | | | | | | |
| Decreased maternal weight gain (g) | 1,2,4-TMB | Female | 131 ± 33 (n = 24) | 124 ± 18 (n = 22) | 126 ± 24 (n = 22) | 116 ± 23 (n = 22) | 95 ± 19** (n = 24) | N/A |
| | 1,3,5-TMB | Female | 135 ± 15 (n = 21) | 138 ± 11 (n = 22) | 118 ± 24* (n = 21) | 95 ± 24** (n = 17) | N/A | 73 ± 28** (n = 18) |
| **Developmental** | | | | | | | | |
| Decreased fetal weight[b] | 1,2,4-TMB | N | 23 | 22 | 22 | 22 | 24 | N/A |
| | | Male | 5.86 ± 0.34 | 5.79 ± 0.30 | 5.72 ± 0.49 | 5.55 ± 0.48* | 5.20 ± 0.42** | N/A |
| | | Female | 5.57 ± 0.33 | 5.51 ± 0.31 | 5.40 ± 0.45 | 5.28 ± 0.40* | 4.92 ± 0.40** | N/A |
| | 1,3,5-TMB | N | 21 | 22 | 21 | 17 | N/A | 18 |
| | | Male | 5.80 ± 0.41[b,c] | 5.76 ± 0.27 | 5.50 ± 0.31 | 5.39 ± 0.55* | N/A | 5.10 ± 0.57** |
| | | Female | 5.50 ± 0.32 | 5. 47 ± 0.21 | 5.27 ± 0.47 | 5.18 ± 0.68 | N/A | 4.81 0.45** |

*$p < 0.05$.
**$p < 0.01$.
[a]Values are expressed as mean ± 1 SD.
[b]Total number of fetuses examined for 1,2,4-TMB and 1,3,5-TMB (respectively): 319, 297 (controls); 275, 314 (492 mg/m³), 293, 282 (1,476 mg/m³), 310, 217 (2,952 mg/m³), 342 (1,2,4-TMB only, 4,428 mg/m³), and 236 (1,3,5-TMB only, 5,904 mg/m³). Number of fetuses per sex was not reported.

Endpoints considered for derivation of the RfC included decreased pain sensitivity (1,2,4-TMB, 1,2,3-TMB), decreased neuromuscular function (1,2,4-TMB, 1,2,3-TMB), decreased red blood cells (RBCs) in male rats (1,2,4-TMB and 1,2,3-TMB), increased white blood cells (WBCs) in male rats (1,2,4-TMB), decreased reticulocytes in female rats (1,2,4-TMB), increased reticulocytes in male rats (1,2,3-TMB), decreased clotting time in female rats (1,2,4-TMB), decreased segmented neutrophils in male and female rats (1,2,3-TMB), increased bronchoalveolar lavage (BAL) total cells in male rats (1,2,4-TMB), increased inflammatory lung lesions in male and female rats (1,2,4-TMB or 1,2,3-TMB), decreased fetal weights in males and female rats (1,2,4-TMB and 1,3,5-TMB), and decreased maternal weight gain (1,2,4-TMB and 1,3,5-TMB) in rats (Saillenfait et al., 2005; Korsak et al., 2000a, b; Korsak et al., 1997; Korsak and Rydzyński, 1996).

Increases in BAL polymorphonuclear leukocytes and lymphocytes observed in the Korsak et al. (1997) study following exposure to 1,2,4-TMB were not selected for possible RfC derivation due to a lack of reporting of exposures at which statistically significant increases occurred. Additionally, Korsak et al. (1997) reported that 123 mg/m³ was the LOAEL for increased BAL total cells, but the

NOAEL for increased BAL macrophages. Therefore, the increase in BAL macrophages was not selected for RfC derivation as this effect was not observed at concentrations that elicited an increase in total BAL cells. Changes in BAL protein and enzyme activity level following 1,2,4-TMB exposure were not selected for RfC derivation due to non-monotonically increasing dose-responses and a lack of corroborating histopathology. A number of endpoints possibly indicating compensatory changes rather than adverse effects were also not selected for RfC derivation: increases in sorbitol dehydrogenase (SDH) following 1,2,4-TMB exposure (Korsak et al., 1997), changes in liver and splenic organ weights and altered clinical chemistry parameters following exposure to 1,2,3-TMB (Korsak et al., 2000b), and changes in serum chemistry parameters in rats exposed to 1,3,5-TMB in a short-term (5 weeks) inhalation study (Wiglusz et al., 1975a). These changes were considered to be possibly compensatory in nature given the lack of accompanying histopathological changes in the relevant organs. Inconsistent temporal patterns of effect were also considered in the decision to not use altered clinical chemistry parameters (Wiglusz et al., 1975a) for the RfC derivation. Increases in reticulocytes in female rats exposed to 1,2,3-TMB (Korsak et al., 2000b) were not selected due to non-monotonicity in response (increases in high concentration animals that were not statistically significant). Increased lymphocytes observed in the same study were excluded from further consideration due to the unusually high standard deviations (SDs) reported in the high-concentration group.

Impaired neuromuscular function and coordination, measured as performance deficits on the rotarod apparatus, was also observed in rats exposed to 1,2,4-TMB or 1,2,3-TMB. The use of rotarod data from Korsak and Rydzyński (1996) was initially considered as a candidate critical effect for these isomers. However, upon critical evaluation of the exposure-response information in the study, it was determined that the endpoint was reported in a manner that reduced the confidence in the observed effect levels. The primary limitation noted for these data relates to the presentation of rotarod performance, which is best represented as a continuous variable, as opposed to a quantal variable such as that presented by Korsak and Rydzyński (1996). In contrast to the percent failures reported by the study authors, the most widely used and accepted measure of rotarod performance in rodents is latency to fall from the rotating rod (Brooks and Dunnett, 2009; Kaspar et al., 2003; Bogo et al., 1981), typically with an arbitrary upper limit on the maximum latency allowed to prevent confounding by fatigue. Although the quantal percent failures data can provide useful hazard information, these measures require an arbitrary selection of the length of time required for successful performance; there is no scientific consensus on an optimal time for this parameter. In addition, when identifying effect levels based on the data presented by Korsak and Rydzyński (1996), latencies on the rod of 1 second versus 119 seconds would be treated identically as failures when, in fact, they indicate very different levels of neurological dysfunction (Bogo et al., 1981). This adds uncertainty when trying to extrapolate to a concentration associated with a minimally adverse effect. Finally, this quantal presentation of data does not allow for interpretations related to intra-rat and intra-group variability in performance. Due to these

reporting limitations, impaired neuromuscular function and coordination, measured as performance deficits on the rotarod apparatus, were considered to be less informative in representing the identified neurotoxicity hazard than the data supporting decreases in pain sensitivity, and thus, were not further evaluated for derivation of the RfC for 1,2,4-TMB.

### 2.1.2. Methods of Analysis for Derivation of Reference Concentrations for TMBs

This assessment uses physiologically based pharmacokinetic (PBPK) model estimates of internal blood dose metrics (for 1,2,4-TMB) and default dosimetric methods (for 1,2,3-TMB and 1,3,5-TMB) coupled with the benchmark dose (BMD) approach to estimate a POD for the derivation of an RfC for TMBs (see Section C.2 of Appendix C and Section D.1 of Appendix D for details regarding PBPK model estimates and BMD modeling, respectively).

The BMD approach involves fitting a suite of mathematical models to the observed dose-response data using the U.S. Environmental Protection Agency (EPA) Benchmark Dose Software (BMDS, version 2.6.0.1). Each fitted model estimates a BMD and its associated 95% lower confidence limit (BMDL) corresponding to a selected benchmark response (BMR). For continuous data (i.e., decreased pain sensitivity, increased BAL total cells, decreased RBCs, decreased reticulocytes, and decreased clotting time) from the Korsak and colleagues studies (Korsak et al., 2000a; Korsak et al., 1997; Korsak and Rydzyński, 1996), and maternal weight gain from Saillenfait et al. (2005), no information is available regarding the change in these responses that would be considered biologically significant. In cases such as this, EPA's *Benchmark Dose Technical Guidance* (U.S. EPA, 2012) recommends against using BMRs based on percent change in the control mean. Therefore, a BMR equal to a 1 SD change in the control mean was used in modeling these endpoints, consistent with EPA's *Benchmark Dose Technical Guidance* (U.S. EPA, 2012). When lacking a biological rationale for setting a BMR, the BMR ultimately chosen should reflect a minimally biologically significant effect level. Although there is some uncertainty surrounding the selection of 1 SD for the BMR (see Section 2.1.6), there is no indication that selecting a lower BMR (i.e., 0.5 SD) would be a more appropriate choice. As such, a BMR equal to 1 SD was used for the endpoints listed above. For the decreased male and female fetal body weight endpoints identified from the Saillenfait et al. (2005) study, a BMR of 5% relative deviation (RD) from the control mean was selected. A 5% decrease in fetal body weight relative to control was determined to be a minimal, biologically significant response. This determination is based on the fact that decreased body weight gain in fetuses and/or pups is considered indicative of altered growth, which has been identified by EPA as one of the four major manifestations of developmental toxicity (U.S. EPA, 1991). In addition, a 10% decrease in adult body weight in animals is generally recognized as a biologically significant response associated with identifying a maximum tolerated dose, but since fetuses and/or pups are generally recognized as a susceptible lifestage, and thus are assumed to be more greatly affected by decreases in body weight than adult animals, a 5% decrease in fetal body weight is considered a biologically significant response. Finally, in humans, reduced birth weight is associated with a series of adverse effects including neonatal and postnatal mortality, coronary

heart disease, arterial hypertension, chronic renal insufficiency, and diabetes mellitus (Barker, 2007; Reyes and Mañalich, 2005). For these reasons, the selection of a BMR of 5% for decreased fetal body weight was considered appropriate. Following BMD modeling, the estimated BMDL is used as the POD for deriving the RfC (see Table 2-6).

The suitability of the above methods to determine a POD is dependent on the nature of the toxicity database for a specific chemical. Some endpoints in the TMB database were not modeled for a variety of reasons, including equivalent responses in the mid and high doses (e.g., increased BAL total cells and decreased reticulocytes [1,2,4-TMB]), responses only in the high exposure group with no changes in responses in lower exposure groups (e.g., increased WBCs [1,2,4-TMB] and decreased RBCs [1,2,3-TMB]), and absence of incidence data (e.g., increased inflammatory lung lesions [1,2,4-TMB and 1,2,3-TMB]). Additionally, some datasets were modeled, but appropriate model fit (to either the mean response or the reported variance) was not achievable for a variety of reasons. Correctly characterizing the variance in a dataset is critical for estimating accurate BMDL values. When a constant (i.e., homogenous) variance model was not able to fit the reported variances, a non-homogenous variance model was used. For example, the reported variances for decreased pain sensitivity (1,2,4-TMB and 1,2,3-TMB) were not homogenous, with variances at 492 mg/m$^3$ being lower or higher (1,2,4-TMB and 1,2,3-TMB, respectively) than reported variances in other dose groups. Fitting a homogenous variance model to these data resulted in poor model fit, and model fit was not improved by running the non-homogenous variance model (see Tables D-2 and D-3). In cases such as this, the high dose was dropped and the models were re-run on the truncated datasets. Dropping the high dose resulted in adequate model fit in some cases (e.g., decreased pain sensitivity; 1,2,4-TMB or 1,2,3-TMB, constant or non-constant variance [respectively], Table D-4), but not others (e.g., decreased clotting time; 1,2,4-TMB). In cases where BMD modeling was not feasible or modeling failed to appropriately describe the dose-response characteristics, the NOAEL/LOAEL approach was used to identify a POD. Detailed modeling methodology and results are provided in Appendix D.

Because an RfC is a toxicity value that assumes continuous human inhalation exposure over a lifetime, data derived from inhalation studies in animals need to be adjusted to account for the non-continuous exposures used in these studies. For 1,2,4-TMB-induced systemic effects (e.g., neurotoxicological effects), the available deterministic PBPK model (Hissink et al., 2007) was used to convert non-continuous external inhalation concentrations (in mg/m$^3$) of 1,2,4-TMB to the internal blood dose metric of average weekly venous blood concentration (in mg/L) of 1,2,4-TMB for Korsak et al. (1997), Korsak et al. (2000a), and Korsak and Rydzyński (1996) only. Weekly average venous blood 1,2,4-TMB concentration was chosen as the internal dose metric on which to base the POD as it is assumed that the parent compound is the toxic moiety of interest and that average venous blood concentration of 1,2,4-TMB adequately represents the target tissue dose across the multiple tissues of interest. The use of concentration of parent compound in venous blood as the relevant dose metric in non-metabolizing, non-first-pass organs is recommended by

Aylward et al. (2011). Furthermore, toluene-induced neurological effects in the brain are provided by Aylward et al. (2011) as an example of a chemically induced toxic endpoint for which this dose metric is relevant. As discussed in Section 1.2.1 (*Mode-of-Action Analysis—Neurotoxic Effects*), 1,2,4-TMB is reasonably expected to have a mode of action for neurotoxic effects similar to toluene, further supporting the selection of venous blood concentration as the relevant internal dose metric. For pulmonary/tracheobronchial effects (e.g., inflammatory lung lesions), the internal dose metric used was the average lung deposition rate per unit surface area (mg/m$^2$/hour, pulmonary + tracheobronchial area).

Although dosimetry can often be nonlinear due to metabolic saturation, and internal dose metrics are expected to correlate more closely to toxic response than external concentrations (McLanahan et al., 2012), the order of analysis employed in this assessment for 1,2,4-TMB is the use of BMD modeling methods with external concentrations used as the dose inputs, followed by conversion of the resulting PODs into human equivalent concentrations (HECs) using the available PBPK model. The order of analysis was conducted in this manner primarily to account for inaccuracies in the animal PBPK model at high exposure concentrations. During the validation and optimization of the animal PBPK model (Hissink et al., 2007) against available animal toxicokinetic datasets, the model accurately reproduced venous blood concentrations of 1,2,4-TMB following repeated (6 hours/day, 5 days/week, 4 weeks) exposures to 123 or 492 mg/m$^3$ (see Section C.2.3.2, Appendix C). However, the PBPK model consistently over-predicted venous blood concentrations following exposure to 1,230 mg/m$^3$. It was concluded that the optimized animal PBPK model produces acceptable simulations of venous blood 1,2,4-TMB concentrations for chronic exposures of up to 100 ppm [492 mg/m$^3$] in rats following inhalation exposure to 1,2,4-TMB (Section C.2.3.2, Appendix C). Therefore, as the model-estimated internal blood dose metrics at the high concentration are not representative of empirically observed blood concentrations, using the high-dose model estimates as dose inputs for BMD modeling is not appropriate. To account for this, BMD modeling was performed on external exposure concentrations in order to identify a POD that was within the validated region of the PBPK model (i.e., <492 mg/m$^3$) and then use the PBPK model to convert that POD into a HEC.

The Hissink et al. (2007) PBPK model was not parameterized for pregnant animals, did not include a fetal compartment, and was not parameterized for either 1,2,3-TMB or 1,3,5-TMB. For these reasons, the model could not be used to account for non-continuous exposures utilized in studies that investigated effects following subchronic exposures to 1,2,3-TMB or 1,3,5-TMB or gestational exposures to 1,2,4-TMB. In order to calculate PODs adjusted for continuous exposures, default dosimetric adjustments were used for these endpoints. For example, in the Saillenfait et al. (2005) study, rats were exposed to 1,2,4-TMB or 1,3,5-TMB for 6 hours/day for 15 consecutive days (gestational days [GDs] 6–20). Therefore, the duration-adjusted PODs for developmental/ maternal effects were calculated as follows:

$$\mathbf{POD_{ADJ}\ (mg/m^3) = POD\ (mg/m^3) \times hours\ exposed\ per\ day/24\ hours}$$

For example, for decreased fetal weight in males following exposure to 1,2,4-TMB, the $POD_{ADJ}$ would be calculated as follows:

**$POD_{ADJ}$ (mg/m³) = 1,640.07 mg/m³ × 6 hours/24 hours**

**$POD_{ADJ}$ (mg/m³) = 410.82 mg/m³**

For subchronic 1,2,3-TMB-induced effects observed in the Korsak et al. (2000b) and Korsak and Rydzyński (1996) studies, rats were exposed to 1,2,3-TMB for 6 hours/day, 5 days/week for 3 months. For these endpoints, the duration-adjusted PODs for effects in rats were calculated as follows:

**$POD_{ADJ}$ (mg/m³) = POD (mg/m³) × hours exposed per day/24 hours × days exposed per week/7 days**

Therefore, for decreased pain sensitivity from Korsak and Rydzyński (1996), the $POD_{ADJ}$ would be calculated as follows:

**$POD_{ADJ}$ (mg/m³) = 97.19 mg/m³× 6 hours/24 hours × 5 days/7 days**

**$POD_{ADJ}$ (mg/m³) = 17.36 mg/m³**

For the derivation of an RfC based upon animal data, the calculated $POD_{ADJ}$ values for 1,2,4-TMB-induced effects were converted to HECs using the available human PBPK model (Hissink et al., 2007) for the selected endpoints from the Korsak and colleagues studies (Korsak et al., 2000a; Korsak et al., 1997; Korsak and Rydzyński, 1996). The human PBPK model was run (as described in Appendix C), assuming a continuous (24 hours/day, 7 days/week) exposure, to estimate a human $POD_{HEC}$ that would result from the same weekly average venous blood concentration or rate of pulmonary deposition reflected in the $POD_{ADJ}$ in animals.

The majority of 1,2,3-TMB-induced subchronic endpoints and all of the 1,2,4-TMB- and 1,3,5-TMB-induced gestational endpoints under consideration for the critical effect result primarily from systemic distribution of the TMB isomers. As the Hissink et al. (2007) PBPK model is not parameterized for 1,2,3-TMB or 1,3,5-TMB, the HECs for subchronic hematological and neurotoxicological 1,2,3-TMB endpoints and maternal/developmental 1,2,4-TMB and 1,3,5-TMB endpoints were calculated by the application of the appropriate dosimetric adjustment factor (DAF) for systemically acting gases with effects distal to the portal of entry, in accordance with the EPA's *RfC Methodology* (U.S. EPA, 1994b). This determination is supported by other factors, including the isomer's relatively low water solubility and non-reactivity. Gases with these properties are expected to preferentially distribute to the lower regions of the respiratory tract where larger surface areas and thin alveolar-capillary boundaries facilitate uptake. Respiratory absorption of 1,2,3-TMB into the bloodstream has been observed to be relatively high (~60%) following inhalation exposures to humans (Järnberg et al., 1996). TMB isomers are also observed to elicit inflammatory lung lesions. Currently, it is not known whether these effects are due to localized deposition or to systemic redelivery of the active agent to the pulmonary region. For the purpose of

deriving reference values, 1,2,3-TMB-induced respiratory endpoints were assumed to be portal-of-entry effects; treating 1,2,3-TMB in such a manner is consistent with HEC calculations for 1,2,4-TMB, in which the dose metric used for respiratory effects was the average lung deposition rate per unit surface area (mg/m$^2$/hour, pulmonary + tracheobronchial area), rather than a dose metric based on venous blood concentrations.

DAFs are ratios of animal and human physiologic parameters, and are dependent on the nature of the contaminant (i.e., particle or gas) and the target site (i.e., respiratory tract or remote to the portal-of-entry [i.e., systemic]) (U.S. EPA, 1994b). For gases with systemic effects, the DAF is expressed as the ratio between the animal and human blood:air partition coefficients:

$$\mathbf{DAF = (Hb/g)_A/(Hb/g)_H}$$

where:

**$(H_{b/}g)_A$ = the animal blood:air partition coefficient and $(H_{b/}g)_H$ = the human blood:air partition coefficient**

For gases that elicit portal-of-entry effects, the DAF is expressed as the ratio between the quotient of ventilation rate divided by respiratory tract surface area for animals and humans, respectively:

$$\mathbf{DAF = (\dot{V}_A/SA_A)/(\dot{V}_H/SA_H)}$$

where:

**$\dot{V}_A$ and $\dot{V}_H$ are the ventilation rates for rats and humans, respectively; and $SA_A$ and $SA_H$ are the surface areas of the tracheobronchial and pulmonary regions in rats and humans, respectively**

Calculations of the isomer-specific DAF values are summarized in Table 2-4.

In cases where the animal blood:air partition coefficient is lower than the human value, resulting in a DAF <1, the calculated value is used for dosimetric adjustments (U.S. EPA, 1994b). When the resulting DAF >1, a default value of 1 is substituted. The calculated $POD_{HEC}$ (mg/m$^3$) values for all endpoints considered for candidate value derivation are presented in Table 2-5.

**Table 2-4. Isomer-specific DAFs using default dosimetric methods**

| | 1,2,4-TMB | 1,2,3-TMB | 1,3,5-TMB |
|---|---|---|---|
| **$(Hb/g)_A$[a]** | 57.7 | 62.6 | 55.7 |
| **$(Hb/g)_H$[b]** | 59.1 | 66.5 | 43 |
| **Systemic DAF** | 0.98 | 0.94 | 1.3 |
| **$\dot{V}_A$ (L/min)** | – | 0.178 (M); 0.125 (F)[c] | – |
| **$\dot{V}_H$ (L/min)** | – | 8.28[d] | – |
| **$SA_A$ ($m^2$)** | – | 0.422 (M); 0.280 (F)[e] | – |
| **$SA_H$ ($m^2$)** | – | 54 (default) | – |
| **Respiratory DAF** | – | 2.75 (M); 2.91 (F) | – |

[a]Derived from Järnberg and Johanson (1995).
[b]Derived from Meulenberg and Vijverberg (2000).
[c]Calculated using the equation $\ln(\dot{V})=b_0 + b_1\ln(BW)$. Body weight (BW) was the average of all body weights in Korsak et al. (2000b) for males (0.404 kg) and females (0.263). Values for $b_0$ (−0.578) and $b_1$ (0.821) for rats were drawn from the 1994 RfC Guidance (U.S. EPA, 1994b). Values for total ventilation (M = 0.267, F = 0.187) were multiplied by (2/3) to calculate alveolar ventilation.
[d]Calculated in L/minute from the value of 0.497 $m^3$/hour used in 1,2,4-TMB PBPK modeling.
[e]Calculated by scaling $BW^{0.95}$.

**Table 2-5. Summary of derivation of PODs for TMBs**

| Endpoint | Isomer | Sex | Model; BMR | POD[a] | $POD_{ADJ}$ | $POD_{HEC}$ |
|---|---|---|---|---|---|---|
| **Neurological** | | | | | | |
| Decreased pain sensitivity | 1,2,4-TMB | Male | Linear, 1 SD | 140.54 | 0.099[b] | 18.15[e] |
| | 1,2,3-TMB | Male | Linear, 1 SD | 97.19 | 17.36[c] | 16.31[f] |
| **Hematological** | | | | | | |
| Decreased RBCs | 1,2,4-TMB | Male | Exponential 2, 1 SD | 451.51 | 0.737[b] | 116.24[e] |
| | 1,2,3-TMB | Male | NOAEL | 523.00 | 93.39[c] | 87.79[f] |
| Increased WBCs | 1,2,4-TMB | Male | NOAEL | 492.00 | 0.868[b] | 131.60[e] |
| Decreased segmented neutrophils | 1,2,3-TMB | Male | Exponential 2, 1 SD | 534.81 | 95.50[c] | 89.77[f] |
| | | Female | Hill, 1 SD | 99.21 | 17.72[c] | 16.65[f] |
| Decreased reticulocytes | 1,2,4-TMB | Female | NOAEL | 492.00 | 0.889[b] | 133.85[e] |
| Increased reticulocytes | 1,2,3-TMB | Male | Linear, 1 SD | 652.80 | 116.57[c] | 109.58[f] |
| Decreased clotting time | 1,2,4-TMB | Female | NOAEL | 129.00 | 0.1335[b] | 24.30[e] |
| **Respiratory** | | | | | | |
| Inflammatory lung lesions | 1,2,4-TMB | Male | NOAEL | 129.00 | 0.564[d] | 61.65[g] |
| | 1,2,3-TMB | Female | NOAEL | 128.00 | 22.86[c] | 66.56[f] |
| Increased BAL total cells | 1,2,4-TMB | Male | LOAEL | 123.00 | 0.533[d] | 58.21[g] |
| **Developmental** | | | | | | |
| Decreased fetal weight | 1,2,4-TMB | Male | Linear, 5% RD | 1,640.07 | 410.02[c] | 401.82[f] |
| | | Female | Linear, 5% RD | 1,612.89 | 403.22[c] | 395.16[f] |
| | 1,3,5-TMB | Male | NOAEL | 1,471.00 | 367.75[c] | 367.75[f] |
| | | Female | NOAEL | 2,974.00 | 743.50[c] | 743.50[f] |
| **Maternal** | | | | | | |
| Decreased maternal weight | 1,2,4-TMB | Female | Polynomial 3, 1 SD | 3,094.13 | 773.53[c] | 758.06[f] |
| | 1,3,5-TMB | Female | NOAEL | 497.00 | 124.25[c] | 124.25[f] |

[a]External air concentration ($mg/m^3$).
[b]Average venous blood concentration (mg/L) predicted for the rat using the PBPK model, given a 6-hour/day, 5-day/week exposure.
[c]Duration-adjusted external concentrations ($mg/m^3$).
[d]Average lung deposition rate per unit surface area ($mg/m^2$/hour, pulmonary + tracheobronchial region), predicted for the rat using the available PBPK model, given a 6-hour/day, 5-day/week exposure.
[e]Continuous exposure level ($mg/m^3$) for a 70-kg human predicted to result in a blood concentration equal to $POD_{adj}$, calculated with PBPK model.
[f]Continuous exposure level ($mg/m^3$) for a human as calculated using default dosimetric methods.
[g]HEC ($mg/m^3$) = $POD_{adj} \times SA_{hum} / Vent_{hum}$, where $SA_{hum}$ = 54.32 $m^2$ (pulmonary + tracheobronchial in a 70-kg adult) and $Vent_{hum}$ = 0.497 $m^3$/hour (total ventilation for a 70-kg adult).

### 2.1.3. Derivation of Candidate Inhalation Values for TMBs

Under EPA's *A Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002), also described in the Preamble, five possible areas of uncertainty and variability were considered in deriving the candidate values for TMBs. An explanation of these five possible areas of

uncertainty and variability and the values assigned to each as a designated UF to be applied to the candidate $POD_{HEC}$ are as follows.

An interspecies uncertainty factor, $UF_A$, of 3 ($10^{1/2}$ = 3.16, rounded to 3) was applied to account for uncertainty in characterizing the toxicokinetic and toxicodynamic differences between rats and humans following inhalation exposure to TMB isomers. In this assessment, the use of a 1,2,4-TMB PBPK model to convert internal doses in rats to administered doses in humans reduces toxicokinetic uncertainty in extrapolating from the rat to humans for that isomer. For 1,2,3-TMB and 1,3,5-TMB, the use of default dosimetric methods to extrapolate external concentrations from rats to humans reduces the toxicokinetic uncertainty for those isomers to the same degree. However, neither of these methods accounts for the possibility that humans may be more sensitive to TMB due to interspecies differences in toxicodynamics. Therefore, a $UF_A$ of 3 was applied to account for this remaining toxicodynamic and any residual toxicokinetic uncertainty not accounted for by application of the PBPK model or default dosimetric methods.

An intraspecies uncertainty factor, $UF_H$, of 10 was applied to account for potentially susceptible individuals in the absence of data evaluating variability of response in the human population following inhalation of TMB isomers. No information is currently available to predict potential variability in human susceptibility, including variability in the expression of enzymes involved in TMB metabolism. However, PBPK modeling on other methylated benzene derivatives (i.e., toluene and xylene) in order to derive chemical-specific $UF_{H\text{-}PK}$ values (the portion of the $UF_H$ accounting for toxicokinetic uncertainty) resulted in $UF_{H\text{-}PK}$ values ranging from 0.96−0.52 for sensitive adults to 0.5−3.9 for extrapolating to children. Both values indicate that the $UF_{H\text{-}PK}$ of 3.16 is sufficient to account for uncertainty due to toxicokinetic differences between possible sensitive subpopulations and the general population (Nong et al., 2006; Pelekis et al., 2001). However, this information does not inform how toxicodynamics may differ between possible sensitive subpopulations and the general population. As such, a $UF_{H\text{-}TD}$ of 3.16 is used, resulting in a total $UF_H$ of 10 to account for potentially susceptible individuals.

A LOAEL-to-NOAEL uncertainty factor, $UF_L$, of 1 was applied because the current approach is to address this factor as one of the considerations in selecting a BMR for BMD modeling. In other words, when selecting a BMR value, care should be taken to select a response level that constitutes a minimal, biologically significant change so that the estimated BMDLs can be assumed to conceptually correspond to a NOAEL. In the case of TMBs, BMRs were preferentially selected based on biological information on what constitutes a biologically significant change for these effects, when such information was available. For example, a 5% reduction in fetal body weight was selected as the BMR for that endpoint based on the fact that a 10% reduction in adult body weight is considered adverse, the assumption that fetuses are a susceptible population and thus more vulnerable to body weight changes, and the fact that decreases in fetal weight in humans are associated with a number of chronic diseases such as hypertension and diabetes. For endpoints for which there was no information available to make assumptions about what constitutes a minimal,

biologically significant response, a BMR equal to a 1 SD change in the control mean was selected. For endpoints that could not be modeled, a $UF_L$ of 1 was applied as a NOAEL was used, except for increased BAL cells following 1,2,4-TMB exposure, to which a UF of 10 was applied due to the use of a LOAEL for this endpoint.

A subchronic-to-chronic uncertainty factor, $UF_S$, of 3 was applied to account for extrapolation from a subchronic exposure duration study to derive a chronic RfC for all endpoints except decreases in fetal weight, to which a $UF_s$ of 1 was applied. The 3-fold UF is applied to the POD identified from the subchronic studies on the assumption that effects observed in a similar study utilizing a chronic exposure duration would be observed at lower concentrations for a number of possible reasons, including potential cumulative damage occurring over the duration of the chronic study or an increase in the magnitude or severity of effect with increasing duration of exposure. Given that the adaptive responses of the nervous system appear to be impaired several weeks after short-term exposure, including prolongation of decreased pain sensitivity phenotypes following environmental challenge using a footshock, there is concern that chronic exposures may more thoroughly overwhelm adaptive responses in the nervous system, and thus lead to more severe responses, compared to shorter-duration exposures. In addition, there is some evidence that neurotoxicity worsens with continued exposure, and thus, effects are expected to be more severe following chronic exposure. For example, decrements in rotarod function were shown to increase in magnitude as a function of exposure duration, worsening from 4 to 8 weeks of exposure (1,2,3-TMB and 1,2,4-TMB), and worsening further from 8 to 13 weeks of exposure (1,2,3-TMB only) (Korsak and Rydzyński, 1996). Although a similar time-course is not available for reduced pain sensitivity, reduced pain sensitivity is observed at approximately 5-fold lower concentrations following subchronic exposure, as compared to acute exposure (see discussion in Section 1.2.1). However, there does not seem to be an exacerbation of other neurotoxic effects at lower doses when comparing subchronic exposures to short-term exposures. Further, evidence from toxicokinetic studies indicates that blood and organ concentrations of TMBs are similar following repeated versus acute exposures (approximately 600 versus 6 hours, respectively; see Table C-9) and the PBPK model predicts less than a 5% increase between the first day and subsequent days of repeated exposures. By extension, it can be reasonably assumed that TMB isomers would not accumulate to an appreciably greater degree following a longer chronic exposure and thus may not lead to effects at lower doses compared to shorter-duration studies. Taken together, the toxicokinetic and toxicological data support the application of a $UF_S$ of 3 for neurotoxic, hematological, and respiratory endpoints. Additionally, for maternal weight gain following exposure to 1,2,4-TMB or 1,3,5-TMB, a $UF_S$ of 3 was applied given that there was no observed decrease in adult body weights in rats exposed to either 1,2,4-TMB or 1,2,3-TMB for longer durations (i.e., 90 days). For decreases in fetal weight, a $UF_S$ of 1 was applied as the gestational period is presumed to be a critical window of susceptibility and no adjustment for duration of exposure is necessary.

A database uncertainty factor, $UF_D$, of 3 ($10^{1/2}$ = 3.16, rounded to 3) was applied to account for database deficiencies. Strengths of the database include the three well-designed subchronic studies that observe exposure-response effects in multiple systems (nervous, respiratory, and hematological systems) in Wistar rats exposed to 1,2,4-TMB or 1,2,3-TMB via inhalation. A deficiency of the database is the lack of a chronic study for any isomer; however, this particular data gap is accounted for in the $UF_S$. An additional strength of the database is the well-designed developmental toxicity study that investigated standard measures of maternal and fetal toxicity in a different strain of rat (Sprague-Dawley) following exposure to 1,2,4-TMB or 1,3,5-TMB. Supporting the observation of developmental toxicity following exposure to TMB isomers, is the observation that exposure to mixtures containing TMB isomers or related compounds (the C9 fraction, Aromatol, toluene, xylene, or ethylbenzene) also elicits developmental toxicity (increased fetal death, decreased fetal weight, cleft palate) in rats, mice, and rabbits (Mckee et al., 1990; Ungvary and Tatrai, 1985), albeit at doses ≥500 mg/m$^3$, which is higher than the lowest LOAEL for neurotoxicity effects in rats (i.e., 123 mg/m$^3$ for decreased pain sensitivity following exposure to 1,2,3-TMB). However, the lack of a multi-generation reproductive toxicity study for TMB isomers is a possible deficiency in the TMB database. There is suggestive evidence that exposure to the C9 fraction may produce reproductive toxicity: exposure of rats to 4,059 mg/m$^3$ TMB isomers (1,500 ppm C9 fraction, containing ~55% TMB isomers) resulted in decreased male fertility (Mckee et al., 1990). Additionally, there was a possible intergenerational effect on body weight in which decreases in fetal/pup/adult weight occurred at lower doses in later generations compared to earlier ones. However, the lowest concentration of TMB isomers (as part of the total mixture) that resulted in decreased body weights was 1,353 mg/m$^3$. Therefore, while reproductive toxicity and progressive, intergenerational health effects appear to be a concern following exposure to mixtures containing TMB isomers, the effects observed occur at concentrations much greater than TMB concentrations that elicit neurotoxicity and hematological effects in adult animals (1,353 mg/m$^3$ versus 123 mg/m$^3$ [1,2,3-TMB] and 492 mg/m$^3$ [1,2,4-TMB]), somewhat reducing the level of concern for observing reproductive effects at much lower concentrations in a multi-generational study.

The lack of a developmental toxicity study for any individual isomer is also a potential weakness of the database. EPA's *A Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002) recommends that the $UF_D$ take into consideration whether there is concern from the available toxicology database that the developing organism may be particularly susceptible to effects in specific organs/systems. TMBs (unspecified isomer) are able to cross the placenta (Cooper et al., 2001; Dowty et al., 1976); therefore, as neurotoxicity is observed in adult animals following exposure to any TMB isomer, there is the concern that exposure to TMB isomers may result in neurotoxicity in the developing organism. EPA's *Guidelines for Neurotoxicity Risk Assessment* (U.S. EPA, 1998) identifies specific effects observed in adult animals (e.g., cognitive and motor function) that can also affect the developing organism exposed in utero. The *Guidelines for*

*Neurotoxicity Risk Assessment* (U.S. EPA, 1998) also indicate that neurotoxicants may have greater access to the nervous system in developing organisms due to an incomplete blood-brain barrier and immature metabolic detoxifying pathways. Lastly, EPA's *A Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002) also states that effects that may be mild or reversible in adults may produce more robust or permanent effects in offspring following developmental exposures. Therefore, there is some concern that the lack of a developmental neurotoxicity study is a major deficiency in the database and that inclusion of such a study would potentially result in a lower POD than the POD for neurotoxicity identified from the available adult TMB toxicity database. Although TMB-specific studies are lacking, inferences can be drawn from developmental studies of related chemicals. Unfortunately, much of the human and animal literature demonstrating the developmental neurotoxicity of related alkylbenzenes comes from epidemiological studies of inhalant abuse or animal studies using exposure paradigms intended to approximate inhalant abuse patterns (i.e., high exposure concentrations and intermittent and non-continuous exposures) (Bowen and Hannigan, 2006; Hass et al., 1999; Hougaard et al., 1999; Hass et al., 1997; Jones and Balster, 1997; Hass et al., 1995), which are difficult to interpret in the context of an RfC. However, information from the related compound, toluene, indicates that, while toluene is able to cross the placenta, and that toluene levels in the placenta, amniotic fluid, and fetal brains increased with increasing exposure concentrations, concentrations of toluene in the amniotic fluid were less than those in maternal blood (Hannigan and Bowen, 2010). Although this fails to account for potential differences in sensitivity of the developing organism to induced effects, or for differences in metabolism, it does suggest that gestational exposure to TMBs might result in lower exposure concentrations to the fetus, which raises uncertainty in the TMB and related compound database regarding whether sufficient amounts of the toxic agent crosses the placenta to elicit effects, and whether the concentrations necessary to elicit effects are lower than those that result in neurotoxicity in the adult organism. In contrast to this, there is evidence from perinatal toxicity studies in rats that low-level exposures to toluene (as low as 5 ppm) early in life (postnatal days [PNDs] 4–12) may disrupt developmental processes that began early in embryogenesis (synaptogenesis, myelination, etc.) and result in neurotoxicity (decrements in spatial learning) (Win-Shwe et al., 2010; Win-Shwe and Fujimaki, 2010). Additionally, evidence of measures of neurotoxicity in children born to occupationally exposed women exists (Grandjean and Landrigan, 2014; Hannigan and Bowen, 2010; Grandjean and Landrigan, 2006) and analogies drawn between related alkylbenzenes and TMBs illustrate reasonable modes of action for developmental neurotoxicity (see Section 1.2.1). Therefore, concern still exists regarding the possibility that exposure to TMB may result in developmental neurotoxicity, potentially at lower TMB concentrations and, as such, a 3-fold $UF_D$ was applied to account for the lack of a developmental neurotoxicity study in the available database for TMB isomers.

Table 2-6 is a continuation of Table 2-5, and summarizes the application of UFs to each POD to derive a candidate inhalation value for each data set. The candidate values presented in

Table 2-6 are preliminary to the derivation of the organ/system-specific RfCs for TMBs. These candidate values are considered individually in the selection of a representative RfC for a specific hazard and subsequent overall RfC for all three of the TMB isomers. Figure 2-1 presents graphically these candidate values, UFs, and PODs, with each bar corresponding to one data set described in Tables 2-5 and 2-6.

**Table 2-6. Summary of derivation of candidate inhalation values for TMBs**

| Endpoint | Isomer | Sex | HEC ($mg/m^3$)[a] | $UF_A$ | $UF_H$ | $UF_L$ | $UF_S$ | $UF_D$ | Composite UF | Candidate value ($mg/m^3$)[a] |
|---|---|---|---|---|---|---|---|---|---|---|
| **Neurological** | | | | | | | | | | |
| Decreased pain sensitivity | 1,2,4-TMB | Male | 18.15 | 3 | 10 | 1 | 3 | 3 | 300 | $6.05 \times 10^{-2}$ |
| | 1,2,3-TMB | Male | 16.31 | 3 | 10 | 1 | 3 | 3 | 300 | $5.44 \times 10^{-2}$ |
| **Hematological** | | | | | | | | | | |
| Decreased RBCs | 1,2,4-TMB | Male | 116.24 | 3 | 10 | 1 | 3 | 3 | 300 | $3.87 \times 10^{-1}$ |
| | 1,2,3-TMB | Male | 87.79 | 3 | 10 | 1 | 3 | 3 | 300 | $2.93 \times 10^{-1}$ |
| Increased WBCs | 1,2,4-TMB | Male | 131.60 | 3 | 10 | 1 | 3 | 3 | 300 | $4.39 \times 10^{-1}$ |
| Increased segmented neutrophils | 1,2,3-TMB | Male | 89.77 | 3 | 10 | 1 | 3 | 3 | 300 | $2.99 \times 10^{-1}$ |
| | | Female | 16.65 | 3 | 10 | 1 | 3 | 3 | 300 | $5.55 \times 10^{-2}$ |
| Decreased reticulocytes | 1,2,4-TMB | Female | 133.85 | 3 | 10 | 1 | 3 | 3 | 300 | $4.46 \times 10^{-1}$ |
| Increased reticulocytes | 1,2,3-TMB | Male | 109.58 | 3 | 10 | 1 | 3 | 3 | 300 | $3.65 \times 10^{-1}$ |
| Decreased clotting time | 1,2,4-TMB | Female | 24.30 | 3 | 10 | 1 | 3 | 3 | 300 | $8.10 \times 10^{-2}$ |
| **Respiratory** | | | | | | | | | | |
| Inflammatory lung lesions | 1,2,4-TMB | Male | 61.65 | 3 | 10 | 1 | 3 | 3 | 300 | $2.06 \times 10^{-1}$ |
| | 1,2,3-TMB | Female | 66.56 | 3 | 10 | 1 | 3 | 3 | 300 | $2.22 \times 10^{-1}$ |
| Increased BAL total cells | 1,2,4-TMB | Male | 58.21 | 3 | 10 | 10 | 3 | 3 | 3,000[b] | N/A |
| **Developmental** | | | | | | | | | | |
| Decreased fetal weight | 1,2,4-TMB | Male | 401.82 | 3 | 10 | 1 | 1 | 3 | 100 | 4.02 |
| | | Female | 395.16 | 3 | 10 | 1 | 1 | 3 | 100 | 3.95 |
| | 1,3,5-TMB | Male | 367.75 | 3 | 10 | 1 | 1 | 3 | 100 | 3.68 |
| | | Female | 743.50 | 3 | 10 | 1 | 1 | 3 | 100 | 7.44 |
| **Maternal** | | | | | | | | | | |
| Decreased maternal weight | 1,2,4-TMB | Female | 758.06 | 3 | 10 | 1 | 3 | 3 | 300 | 2.53 |
| | 1,3,5-TMB | Female | 124.25 | 3 | 10 | 1 | 3 | 3 | 300 | $4.14 \times 10^{-1}$ |

[a]As calculated by application of UFs, not rounded to 1 significant digit.
[b]Endpoint excluded from further consideration due to increased uncertainty relative to other endpoints (as illustrated by a composite UF 10-fold higher than any other endpoint.

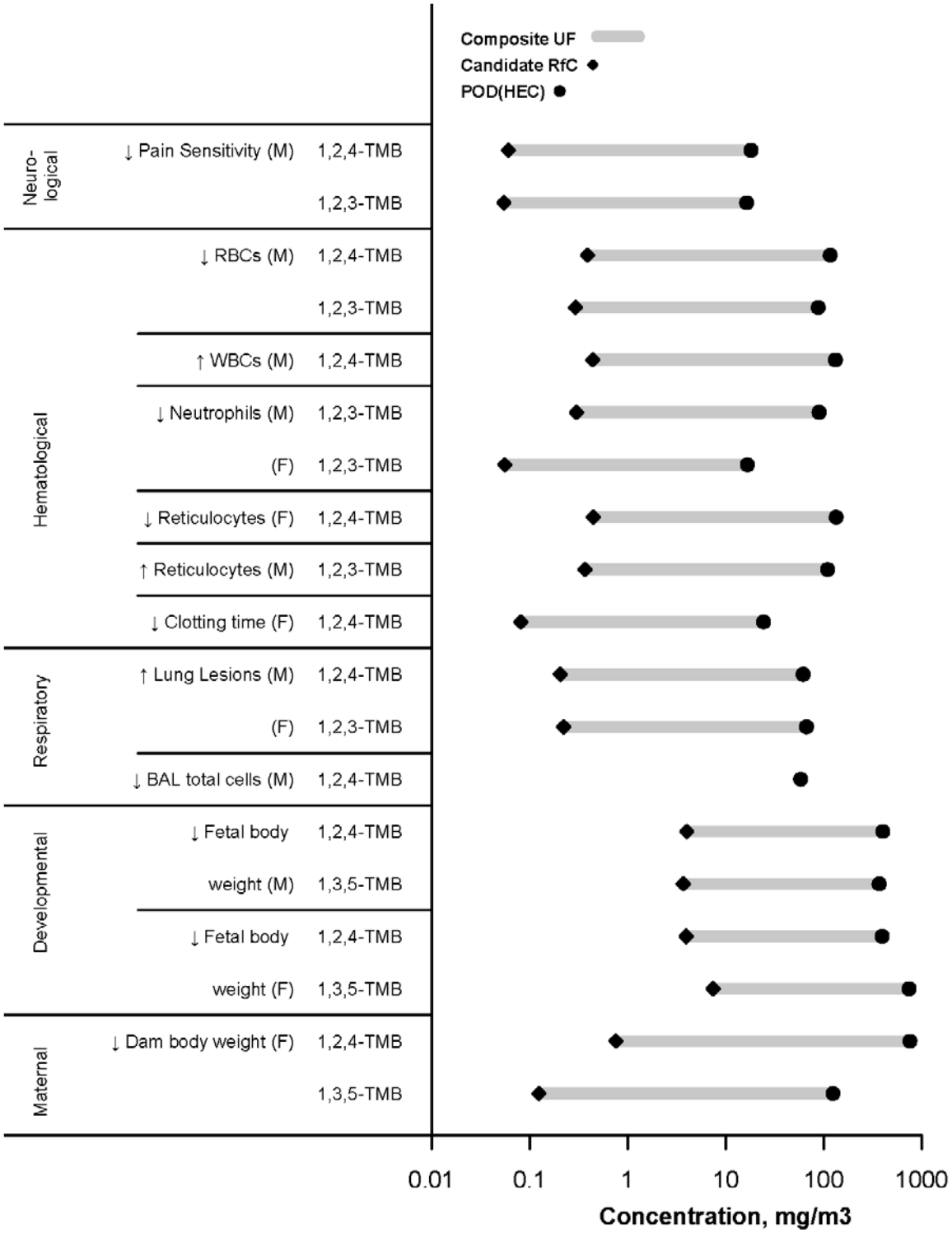



**Figure 2-1. Candidate values with corresponding POD and composite UF for TMBs**

### 2.1.4. Derivation of Organ/System-Specific Reference Concentrations for TMBs

Table 2-7 distills the candidate values from Table 2-6 into a single value for each isomer, where possible, for each organ or system. The single isomer-specific RfC selected for a particular organ/system was preferably chosen using biological and toxicological information regarding that endpoint. If no compelling biological information exists on which to base the selection, the lowest RfC for that organ/system was selected. These organ- or system-specific RfCs may be useful for subsequent cumulative risk assessments that consider the combined effect of multiple agents acting at a common site. In the TMBs Toxicological review, a $UF_D$ of 3 is applied to derive each candidate value to account for the lack of a developmental neurotoxicity study in the TMB database. Cumulative risk assessments based on endpoints other than developmental toxicity should consider whether the $UF_D$ of 3 is appropriate for the specific endpoint being assessed. How organ-specific RfCs are used will be situation-specific and should ultimately fit the needs of the program using them.

The individual organs and systems for which specific RfC values were derived were the neurological, hematological, and respiratory systems, along with specific RfCs derived for the pregnant animal (maternal) and developing fetus (developmental). The RfC values for the neurological system, based on decreased pain sensitivity following exposure to 1,2,4-TMB or 1,2,3-TMB, were similar ($6 \times 10^{-2}$ versus $5 \times 10^{-2}$ mg/m$^3$, respectively). The RfC for 1,2,4-TMB was selected for the overall RfC for all TMBs (see Section 2.1.5 for details). The RfC values for the hematological system (based on decreased clotting time for 1,2,4-TMB and decreased segmented neutrophils for 1,2,3-TMB) were similar to those calculated for neurological effects. The respiratory RfCs were somewhat higher compared to the neurological and hematological RfCs: $2 \times 10^{-1}$ mg/m$^3$. This generally indicates that effects in all of these systems may be of concern at similar levels of environmental exposure. Decisions concerning averaging exposures over time for comparison with the developmental RfC should consider the lifestages of concern. For example, fluctuations in exposure levels that result in elevated exposures during gestation could potentially lead to an appreciable risk, even if average levels over the full exposure duration were less than or equal to the RfC. Alternatively, developmental toxicity may not be a concern during exposure scenarios in which exposure is occurring outside of the critical window of development.

**Table 2-7. Organ/system-specific RfCs and overall RfC for TMBs**

| Effect | Isomer | Basis | RfC ($mg/m^3$) | Composite UF | Exposure description | Confidence |
|---|---|---|---|---|---|---|
| Neurological | 1,2,4-TMB | Decreased pain sensitivity | $6 \times 10^{-2}$ | 300 | Subchronic | Low to medium |
| | 1,2,3-TMB | | $5 \times 10^{-2}$ | 300 | Subchronic | Low to medium |
| Hematological | 1,2,4-TMB | Decreased clotting time | $8 \times 10^{-2}$ | 300 | Subchronic | Low to medium |
| | 1,2,3-TMB | Decreased segmented neutrophils | $6 \times 10^{-2}$ | 300 | Subchronic | Low to medium |
| Respiratory | 1,2,4-TMB | Inflammatory lung lesions | $2 \times 10^{-1}$ | 300 | Subchronic | Low to medium |
| | 1,2,3-TMB | | $2 \times 10^{-1}$ | 300 | Subchronic | Low to medium |
| Developmental | 1,2,4-TMB | Fetal weight | 4 | 100 | Gestational | Low to medium |
| | 1,3,5-TMB | | 4 | 100 | Gestational | Low to medium |
| Maternal | 1,2,4-TMB | Decreased maternal weight | 3 | 300 | Subchronic | Low to medium |
| | 1,3,5-TMB | | $4 \times 10^{-1}$ | 300 | Subchronic | Low to medium |
| **Overall RfC (Neurological)** | **All TMB isomers** | **Decreased pain sensitivity** | **$6 \times 10^{-2}$** | **300** | **Subchronic** | **Low to medium** |

### 2.1.5. Selection of the Overall Reference Concentration for TMBs

Neurotoxicity is the most consistently observed endpoint in the toxicological database for TMBs. According to EPA's *Guidelines for Neurotoxicity Risk Assessment* (U.S. EPA, 1998), many neurobehavioral changes reported in the available TMB studies are regarded as adverse, and, in particular, decreased pain sensitivity, measured as an increased latency to paw-lick in hot plate tests, represents an alteration in neurobehavioral function (U.S. EPA, 1998). It is important to consider that the choice of the critical effect is intended as a representation of the overall neurotoxic hazard of TMB, which, as discussed in Section 1.2.1, is not limited to observations of reduced pain sensitivity in only one study. Rather, the TMB database includes the observations of multiple correlated and replicated measures of neurotoxicity, which in turn, strengthen the overall evidence for a neurotoxic hazard. Decreased pain sensitivity (Korsak and Rydzyński, 1996) or decreased pain sensitivity following a footshock challenge was observed in multiple studies across multiple exposure durations (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998; Gralewicz et al., 1997b), and in the presence of other measures of altered neurobehavior, including impaired neuromuscular function and altered cognitive function. Additionally, neurological symptoms (e.g., hand tremble, weakness) were observed in worker populations exposed to complex VOC mixtures containing TMB isomers. Notably, pain sensitivity has not been evaluated in any occupational epidemiologic or controlled human exposure studies. However, other measures of neurotoxicity were noted in human populations, including altered

neuromuscular function (hand tremble, altered motor control), altered cognition (memory problems), and increased nervousness and/or anxiety, suggesting a consistency and coherency of neurotoxic effects in humans and animals following exposure to TMBs. This supports the assumption that neurotoxic effects observed in animals, but not yet evaluated in human populations, are relevant to human populations.

Although a superficial consideration of the available evidence may lead to a determination that decreased pain sensitivity may be a reversible endpoint related to intoxication and the presence of TMB isomers in the body, consideration of the neurotoxicological database as a whole supports the conclusion that decreased pain sensitivity is a persistent effect lasting up to 2 months post exposure (see Section 1.2.1, *Summary of Neurological Effects*, for more details). Additionally, although it is important to consider the potential for reversibility of neurological effects, "for chronic lifetime exposures, designation of an effect as irreversible or reversible is academic, as exposure is presumed to be lifetime (i.e., there is no post-exposure period)" (U.S. EPA, 2002) (pg. 3-27). In other words, the nature of an RfC precludes the possibility of recovery of the critical effect. This supports the choice of the principal study even when all aspects of the pain sensitivity phenotype were identified as transient, which, notably, does not appear to be the case. Ultimately, a consideration of the entire body of evidence for decreased pain sensitivity supports the conclusion that exposure to TMB isomers elicits a long-lasting, non-reversible insult to the central nervous system (CNS).

Taken as a whole, the database supports the characterization of decreased pain sensitivity associated with exposure to TMBs as relevant to human health. Given the consistency of observations from hot plate tests with or without footshock challenge across several studies from the same research group using multiple durations of exposure in male Wistar rats, the abundant supportive evidence of neurotoxic effects at similar concentrations across a range of behavioral measures and exposure durations, as well as the evidence and biological plausibility of similarities in neurological effects between rats and humans, there is strong evidence that neurotoxicity is the primary hazard associated with exposure to TMB isomers. Based on the above considerations, Korsak and Rydzyński (1996) was selected as the principal study for derivation of the RfC for TMBs, and decreased pain sensitivity measured immediately after subchronic exposure is identified as an adverse neurotoxic effect and is thus an appropriate critical effect on which to base the RfC. Unfortunately, there are no subchronic data pertaining to the neurotoxicity of 1,3,5-TMB. However, the available evidence regarding toxicokinetic and toxicological similarities between the isomers supports the assumption that decreased pain sensitivity is also a concern of exposure to 1,3,5-TMB. Additionally, although decreased pain sensitivity was observed in multiple short-term studies (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Wiaderna et al., 1998; Gralewicz et al., 1997b), those studies used a shorter exposure duration of 28 days and were determined to be less suitable for the derivation of chronic RfCs (as stated above). However, these studies qualitatively contribute to the determination that neurotoxicity is the most consistently observed effect in the

TMB toxicity database, and support the determination that Korsak and Rydzyński (1996) is the most appropriate principal study for derivation of the RfC, as it is the only subchronic study with quantitative data appropriate for dose-response evaluation investigating the neurotoxic effects of individual TMB isomers. Therefore, the RfC for neurotoxicity, based on decreased pain sensitivity from Korsak and Rydzyński (1996), was selected as the RfC for TMBs.

A PODHEC of 18.15 mg/m$^3$ for decreased pain sensitivity following exposure to 1,2,4-TMB (Korsak and Rydzyński, 1996) was used as the POD from which to derive the chronic RfC for TMBs (see Table 2-6). The $POD_{HEC}$ for 1,2,4-TMB was selected over that of 1,2,3-TMB due to increased confidence in that value given that it was calculated via the application of a validated PBPK model, whereas the 1,2,3-TMB value was estimated using default dosimetric methods. However, this distinction is largely academic, as the candidate RfCs for the two isomers are almost identical ($6 \times 10^{-2}$ versus $5 \times 10^{-2}$ mg/m$^3$, respectively) after application of UFs. The UFs, selected and applied in accordance with the procedures described in EPA's *A Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002) (Section 4.4.5 of the report), were discussed previously in Section 2.1.3. Application of the **composite UF of 300** to the $POD_{HEC}$ yields the following chronic RfC for TMBs:

**$RfC = POD_{HEC} \div UF = 18.15$ mg/m$^3$ $\div$ 300 $= 0.0605$ mg/m$^3$ $= 6 \times 10^{-2}$ mg/m$^3$ (rounded to one significant digit)**

### 2.1.6. Uncertainties in the Derivation of the Reference Concentration for TMBs

As presented above, the UF approach, following EPA practices and RfC guidance (U.S. EPA, 2002, 1994b), was applied to the $POD_{HEC}$ for 1,2,4-TMB in order to derive the chronic RfC for all TMB isomers. Factors accounting for uncertainties associated with a number of steps in the analyses were adopted to account for extrapolation from animals to humans, a diverse human population of varying susceptibilities, duration of exposure, POD determination methodologies (NOAEL, LOAEL, or BMDL), and database deficiencies.

The critical effect selected, decreased pain sensitivity, does not introduce substantial uncertainty into the RfC calculation, as selection of alternative hematological effects would result in RfCs that would be equivalent when rounding to one significant digit (i.e., $6 \times 10^{-2}$ mg/m$^3$, see Figure 2-1). The two isomer-specific RfC values for 1,2,4-TMB and 1,2,3-TMB are identical to one another. While it may seem inconsequential as to which value to ultimately select as the RfC for all TMB isomers, there is increased confidence in the 1,2,4-TMB value regarding its calculation via a PBPK model rather than the use of default dosimetric methods. Additionally, there is some utility in selecting the RfC for 1,2,4-TMB as the final RfC, given its use in ultimately calculating the RfD for TMBs (see Section 2.2.3). There is some uncertainty in adopting this RfC for 1,3,5-TMB in light of the lack of subchronic neurotoxicity data with which to calculate an isomer-specific RfC for 1,3,5-TMB. However, as stated above in Section 2.1.5, 1,3,5-TMB shares many commonalities and similarities with 1,2,4-TMB and 1,2,3-TMB regarding chemical, toxicokinetic, and toxicological

properties that support the adoption of the value of one isomer for the other. The majority of uncertainty regarding 1,3,5-TMB's database involves the lack of a chronic, subchronic, or multi-generational reproductive study for this isomer. Given the similarities in toxicity from the developmental toxicity study, and neurotoxicity and respiratory toxicity observed in the available acute and short-term studies, there is strong evidence that the toxicity resulting from subchronic exposure to any isomer can be expected to be similar. Regarding the selection of the DAF and dose metric for respiratory effects due to 1,2,3-TMB or 1,2,4-TMB exposure (respectively), there is some uncertainty regarding whether these endpoints are due to localized effects or to systemic redelivery of the toxic moiety to the pulmonary region. However, there is evidence that TMBs act as acute respiratory irritants and induce inflammatory responses following longer exposures. These observations support the conclusion that TMB-induced respiratory effects most likely act as portal-of-entry effects within the pulmonary region.

Some uncertainty exists regarding the selection of the BMRs for use in BMD modeling due to the absence of information to determine the biologically significant level of response associated with the endpoints. In cases such as this, the selection of a BMR of 1 SD for continuous endpoints is supported by EPA guidance (U.S. EPA, 2012); using a BMR of 1 SD assumes that a change of that magnitude constitutes a minimally biologically significant response. Using decreased pain sensitivity as an example, there is a lack of information as to what constitutes a biologically significant response and statistical significance testing was not definitive in identifying exposure levels that may be considered biologically significant. Given the uncertainty in whether a change of 1 SD is biologically significant, one option would be to use a BMR equal to 0.5 SD. For decreased pain sensitivity, using 0.5 SD as the BMR would lower the POD by 50%. However, there is no compelling evidence that reducing the BMR to 0.5 SD is necessary and doing so may be overly conservative. Therefore, for the purposes of this assessment, a BMR of 1 SD change is used for endpoints lacking information on what constitutes a biologically significant response.

Uncertainty regarding the selection of particular models for individual endpoints exists, as selection of alternative models could decrease or increase the estimated POD and consequently, the RfC. The selection criteria for model selection was based on a practical approach as described in EPA's *Benchmark Dose Technical Guidance* (U.S. EPA, 2012). Uncertainty may exist in the PBPK model estimates of internal blood dose metrics for the rat, and subsequent HEC calculations for humans, including parameter uncertainty, but such uncertainties would apply equally to all endpoints. Equivalently, any uncertainty regarding default dosimetric adjustments for 1,2,3-TMB and 1,3,5-TMB endpoints would apply equally to all endpoints for each individual endpoint. There is some uncertainty in comparing the isomer-specific RfC values calculated via PBPK methods and default dosimetric methods. However, this uncertainty does not seem to be overly concerning as the two RfC values for 1,2,4-TMB and 1,2,3-TMB are identical to one another. The RfC value for 1,2,4-TMB was ultimately selected for use as the overarching RfC value for TMBs due to slightly

more confidence in the validated PBPK model versus default dosimetric adjustments, and its utility in route-to-route conversions for calculating RfD values.

### 2.1.7. Confidence Statement for the Reference Concentration for TMBs

A confidence level of high, medium, or low is assigned to the study used to derive the RfC, the overall database, and the RfC itself, as described in Section 4.3.9.2 of EPA's *Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry* (U.S. EPA, 1994b).

Confidence in the study from which the critical effect was identified, Korsak and Rydzyński (1996), is low to medium. The study is a peer-reviewed study that utilized three dose groups plus untreated controls, employed an appropriate number of animals per dose group, and performed appropriate statistical analyses. However, sources of uncertainty exist that reduce confidence in this study.

One area of uncertainty regarding this study is the lack of reported actual concentrations. However, as the methods by which the test atmosphere was generated and analyzed were reported in sufficient detail, and given the fact that this laboratory has used this methodology in subsequent studies (Korsak et al., 2000a, b) and achieved appropriate actual concentrations (i.e., within 10% of target concentrations), the concern regarding the lack of reported actual concentrations is minimal. Another source of uncertainty is the fact that the Korsak and Rydzyński (1996) study does not explicitly state that the reported measures of variance in Table 1 of that reference are SDs. However, careful analysis of the reported levels of variance and magnitude of statistical significance reported indicate that the measures of variance are SDs. Supporting this conclusions is the observation that all other papers by Korsak and colleagues (Korsak et al., 2000a, b; Korsak et al., 1997; Korsak et al., 1995) report variance as SDs. The critical effect on which the RfC is based is well-supported, as the weight of evidence for TMB-induced neurotoxicity is coherent across species (i.e., human and rat), coherent across isomers, and consistent across multiple exposure durations (i.e., acute, short-term, and subchronic) and outcome measures (Gralewicz and Wiaderna, 2001; Chen et al., 1999; Wiaderna et al., 1998; Gralewicz et al., 1997b; Gralewicz et al., 1997a; Korsak and Rydzyński, 1996; Norseth et al., 1991).

The database for TMBs includes acute, short-term, subchronic, and developmental toxicity studies in rats and mice. However, confidence in the overall database is low to medium because it lacks chronic and developmental neurotoxicity studies, and the studies supporting the critical effect predominantly come from the same research institute. **The overall confidence in the RfC for TMBs is low to medium.**

### 2.1.8. Calculation of Subchronic Reference Concentrations for TMBs

In addition to providing RfCs for chronic exposures in multiple systems, this document also provides an RfC for subchronic- duration exposures. In the case of TMBs, all of the studies used to calculate the chronic RfCs were subchronic or gestational in duration. Therefore, the method to

calculate subchronic RfCs is identical to that used for the calculation of chronic RfCs, minus the application of a $UF_S$ (see Table 2-8). The individual organs and systems for which specific subchronic RfC values were derived were the neurological, hematological, and respiratory systems, along with specific RfCs derived for the pregnant animal (maternal) and developing fetus (developmental). The subchronic RfC values for the neurological system, based on decreased pain sensitivity following exposure to 1,2,4-TMB or 1,2,3-TMB, were identical ($2 \times 10^{-1}$ mg/m$^3$). The RfC for 1,2,4-TMB was selected for the overall subchronic RfC for TMBs for the reasons outlined in Section 2.1.5. The subchronic RfC values for the hematological system (based on decreased clotting time for 1,2,4-TMB and decreased segmented neutrophils for 1,2,3-TMB) were identical to those calculated for neurological effects. The respiratory subchronic RfCs were somewhat higher compared to the neurological and respiratory RfCs: $6 \times 10^{-1}$ mg/m$^3$. This generally indicates that effects in all of these systems may be of concern at similar levels of environmental exposure. It should be noted that the subchronic RfC values for the developing fetus are identical to the chronic RfCs derived for the developing fetus in Sections 2.1.3 and 2.1.4, as gestation represents a critical window of susceptibility and no $UF_S$ was applied to account for less-than-chronic exposure in either case. The subchronic inhalation RfC is intended for use with exposures for more than 30 days, up to approximately 10% of the lifespan in humans. **The subchronic RfC was set to $2 \times 10^{-1}$ mg/m$^3$ based on neurological effects following exposure to 1,2,4-TMB**.

**Table 2-8. Summary of derivation of subchronic RfC values for TMBs**

| Effect | Isomer | Basis | RfC (mg/m$^3$) | Composite UF | Exposure description | Confidence |
|---|---|---|---|---|---|---|
| Neurological | 1,2,4-TMB | Decreased pain sensitivity | $2 \times 10^{-1}$ | 100 | Subchronic | Low to medium |
| | 1,2,3-TMB | | $2 \times 10^{-1}$ | 100 | Subchronic | Low to medium |
| Hematological | 1,2,4-TMB | Decreased clotting time | $2 \times 10^{-1}$ | 100 | Subchronic | Low to medium |
| | 1,2,3-TMB | Decreased segmented neutrophils | $2 \times 10^{-1}$ | 100 | Subchronic | Low to medium |
| Respiratory | 1,2,4-TMB | Inflammatory lung lesions | $6 \times 10^{-1}$ | 100 | Subchronic | Low to medium |
| | 1,2,3-TMB | | $6 \times 10^{-1}$ | 100 | Subchronic | Low to medium |
| Developmental | 1,2,4-TMB | Fetal weight | 4 | 100 | Gestational | Low to medium |
| | 1,3,5-TMB | | 4 | 100 | Gestational | Low to medium |
| Maternal | 1,2,4-TMB | Decreased maternal weight | 8 | 100 | Subchronic | Low to medium |
| | 1,3,5-TMB | | 1 | 100 | Subchronic | Low to medium |
| **Subchronic Overall RfC (Neurological)** | **All TMB isomers** | **Decreased pain sensitivity** | **$2 \times 10^{-1}$** | **100** | **Subchronic** | **Low to medium** |

## 2.2. ORAL REFERENCE DOSE FOR EFFECTS OTHER THAN CANCER FOR TMBs

The RfD (expressed in units of mg/kg-day) is defined as an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily 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, a LOAEL, or a 95% lower bound on the benchmark dose (BMDL), with UFs generally applied to reflect limitations of the data used.

### 2.2.1. Identification of Studies and Effects for Dose-Response Analysis and Derivation of Reference Doses for TMBs

No chronic or subchronic studies were identified for any isomer of TMB that utilized the oral route of exposure. However, one subchronic study (Adenuga et al., 2014) investigated the oral toxicity of 1,3,5-TMB (this study is a peer-reviewed summary report of the more detailed Koch Industries (1995b) report). This study investigated general toxicity endpoints in rats exposed to 1,3,5-TMB via gavage, including organ weight changes, clinical chemistry parameters, and hematological endpoints. Data from this study pertaining to the hazards observed in animals (hematological toxicity) were considered as a critical effect for the purpose of determining the POD for derivation of the oral RfD for TMB isomers. The Adenuga et al. (2014) study is adequate for dose-response analysis. This study used an appropriate laboratory animal species and appropriate sham-exposed controls. Animals were exposed to 1,3,5-TMB, reported as 99.2% pure (1,2,4-TMB reported as impurity at 0.7% (Koch Industries, 1995b)). This study utilized an appropriate route (gavage) and duration (subchronic) of expose. This study also used a reasonable range of appropriately-spaced exposure levels to facilitate dose-response analysis. An appropriate latency between exposure and development of toxicological outcomes was used, and the persistency of some outcomes after termination of exposure was investigated. Adequate numbers of animals per exposure group were used, and appropriate statistical tests (pair-wise comparisons) were performed. Regarding the reporting of exposure methodology, the study reported actual concentrations in mg/mL for the low and high exposure groups (10 and 120 mg/mL) at the beginning, middle, and end of the exposure period (weekly values given in Koch Industries (1995b)). Actual concentrations were given for weeks 1 and 2 for the middle exposure group (40 mg/mL). No actual dosages in mg/kg were given for any of the exposure groups (expressed as 50, 200, and 600 mg/kg). Although no actual dosage information was given, all of the reported test article concentrations in mg/mL were within 10% of the target concentrations, which increases the confidence in the overall evaluation and adequacy of this study.

This study examined 1,3,5-TMB-induced toxicity in multiple target organs/systems, and any endpoint that demonstrated statistically significant increases or decreases relative to control were

considered for the derivation of the RfD for TMBs. A number of endpoints possibly indicating compensatory changes rather than adverse effects were not considered for the RfD derivation. These endpoints include changes in various clinical chemistry parameters and kidney and liver weights. These changes were considered to be possibly compensatory in nature given the lack of accompanying histopathological changes in the relevant organs. Discounting these endpoints left an observed increase in monocytes in male rats as the only statistically significant effect (0.1 ± 0.09 [0 mg/kg-day], 0.2 ± 0.09 [50 mg/kg-day], 0.3 ± 0.17 [200 mg/kg-day], 0.2 ± 0.18 [600 mg/kg-day], units = $\times 10^6/mm^3$). Although a slight increase in monocytes may be of questionable adversity if taken out of context of the TMB database, a number of endpoints involving the alteration of WBC counts have been observed in the inhalation toxicity database. Given that, it was deemed that the observed increase in monocytes following oral exposures was possibly indicative of an underlying toxicity to the hematological system also evident following inhalation exposure.

### 2.2.2. Methods of Analysis for Derivation of Reference Doses for TMBs

This assessment uses the BMD approach to estimate a POD for the derivation of an RfD for TMBs. The BMD approach involves fitting a suite of mathematical models to the observed dose-response data using EPA's BMDS (version 2.6.0.1). Each fitted model estimates a BMD and its associated BMDL corresponding to a selected BMR. For continuous data (i.e., increased monocytes) from the Adenuga et al. (2014) study, no information is available regarding the change in these responses that would be considered biologically significant; thus, a BMR equal to a 1 SD change in the control mean was used in modeling these endpoints, consistent with EPA's *Benchmark Dose Technical Guidance* (U.S. EPA, 2012). A 1 SD shift in the control mean roughly corresponds to a 10% increase in the number of animals responding adversely (i.e., number of animals either falling below the 2nd percentile or above the 98th percentile for decreasing and increasing responses, respectively) (U.S. EPA, 2012). Thus, using a 1 SD change in response approximates a BMR based on 10% extra risk, partially harmonizing BMR selection for continuous and dichotomous endpoints. The estimated BMDL is then used as the POD for deriving the RfD. The suitability of the above methods to determine a POD is dependent on the nature of the toxicity database for a specific chemical. However, no issues were encountered when modeling the dose-response data for increased monocytes. Detailed modeling methodology and results are provided in Appendix D. For increased monocytes, the best-fitting model was the Exponential 4 model (using non-constant variance), which returned BMD and BMDL values of 51.99 and 13.92 mg/kg-day, respectively. The BMDL value is subsequently used as the POD.

Because an RfD is a toxicity value that assumes continuous human oral exposure over a lifetime, data derived from inhalation studies in animals need to be adjusted to account for the non-continuous exposures used in these studies. In the Adenuga et al. (2014) study, rats were exposed to 1,3,5-TMB via gavage 5 days/week for 90/91 days. Because no PBPK model exists for 1,3,5-TMB, the duration-adjusted POD for increased monocytes in male rats was calculated as follows:

**$POD_{ADJ}$ (mg/kg-day) = POD (mg/kg-day) × days exposed per week/7 days**

Therefore, for increased monocytes from Adenuga et al. (2014), the $POD_{ADJ}$ would be calculated as follows:

**$POD_{ADJ}$ = 13.92 mg/kg-day × 5 days/7 days = 9.94 mg/kg-day**

Because increased monocytes results from systemic distribution of 1,3,5-TMB, and no PBPK model exists for this isomer, the human equivalent dose (HED) for 1,3,5-TMB was calculated by allometrically scaling the $POD_{ADJ}$ by a DAF equal to the ratio of animal and human body weights, scaled to the ¾ power (U.S. EPA, 2011). In other words, the DAF is:

$$\mathbf{DAF = (BW_A/BW_H)^{1-(3/4)}}$$

Where $BW_A$ and $BW_H$ are the animal and human body weights, respectively. At the end of the exposure period, male rats in the Adenuga et al. (2014) study weighed an average of 0.605 kg across all exposure groups (detailed body weights provided in Koch Industries (1995b)); a default human body weight of 70 kg was used. Therefore, the DAF for increased monocytes was:

$$\mathbf{DAF = (0.605/70)^{0.25} = 0.305}$$

Calculation of the $POD_{HEC}$ would then follow as:

**$POD_{HED}$ = $POD_{ADJ}$ × DAF = 9.94 mg/kg-day × 0.305 = 3.03 mg/kg-day**

### 2.2.3. Derivation of the Reference Dose for TMBs

A $POD_{HED}$ of 3.03 mg/kg-day was derived for the oral database using default dosimetric adjustment (allometric scaling by body weight) based on hematological effects (i.e., increased monocytes) observed by Adenuga et al. (2014) following gavage administration. The UFs, selected and applied in accordance with the procedures described in EPA's *A Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002) (Section 4.4.5 of the report), also described in the Preamble, address five possible areas of uncertainty and variability were considered in deriving the candidate oral values for TMBs. An explanation of these five possible areas of uncertainty and variability and the values assigned to each as a designated UF to be applied to the $POD_{HED}$ are as follows.

A $UF_A$ of 3 ($10^{1/2}$ = 3.16, rounded to 3) was applied to account for uncertainty in characterizing the toxicokinetic and toxicodynamic differences between rats and humans following oral exposure to TMB isomers. In this assessment, the use of default dosimetric methods to extrapolate oral doses from rats to humans reduces the toxicokinetic uncertainty for 1,3,5-TMB to the same degree. However, neither of these methods accounts for the possibility that humans may be more sensitive to TMB due to interspecies differences in toxicodynamics. Therefore, a $UF_A$ of 3 was applied to account for this remaining toxicodynamic and any residual toxicokinetic uncertainty not accounted for by application of the default dosimetric methods.

A $UF_H$ of 10 was applied to account for potentially susceptible individuals in the absence of data evaluating variability of response in the human population following inhalation of TMB isomers. No information is currently available to predict potential variability in human susceptibility, including variability in the expression of enzymes involved in TMB metabolism.

A $UF_L$ of 1 was applied because the current approach is to address this factor as one of the considerations in selecting a BMR for BMD modeling. In this case, BMRs were preferentially selected based on biological information on what constitutes a biologically significant change for these effects. When no information was available to make assumptions about what constitutes a minimal, biologically significant response, a BMR equal to a 1 SD change in the control mean was selected.

A $UF_S$ of 3 ($10^{1/2}$ = 3.16, rounded to 3) was applied to account for extrapolation from a subchronic exposure duration study to derive a chronic RfD. The 3-fold UF is applied to the POD identified from the subchronic study on the assumption that effects observed in a similar chronic study would be observed at lower RfD concentrations for a number of possible reasons, including potential cumulative damage occurring over the duration of the chronic study or an increase in the magnitude or severity of effect with increasing duration of exposure. However, evidence from inhalation toxicokinetic studies and PBPK modeling of inhalation data indicate that blood and organ concentrations of TMBs are similar following repeated versus acute exposures (approximately 600 versus 6 hours, respectively; see Table C-9). It is reasonable to assume similar behavior following oral exposures. This reduces, but does not completely remove, the concern that longer oral exposures may result in adverse effects at lower doses. As such, a $UF_S$ of 3 was applied to account for the possibility of a lower POD following chronic exposures.

A $UF_D$ of 3 ($10^{1/2}$ = 3.16, rounded to 3) was applied to account for database deficiencies. Strengths of the database include a single well-designed subchronic study that observed exposure-response effects in Wistar rats exposed to 1,3,5-TMB via gavage. Beyond this study, however, there is a lack of any other studies in the oral TMB database, including no neurotoxicity, developmental neurotoxicity, or reproductive/developmental toxicity studies. The lack of these types of studies is a potentially weakness in the TMB oral database. A chemical-specific deficiency in the oral TMB database is the lack of neurotoxicity studies. Given the ample evidence of neurotoxicity following inhalation exposure to TMB isomers in rats, it is reasonable to expect that neurotoxicity would also be a concern following oral exposures. However, the PODs for hematological effects in the inhalation database (16.65 mg/m$^3$ [decreased segmented neutrophils, 1,2,3-TMB] and 24.30 mg/m$^3$ [decreased clotting time, 1,2,4-TMB]) were similar to the POD for decreased pain sensitivity (18.15 and 16.32 mg/m$^3$ for 1,2,4-TMB and 1,2,3-TMB, respectively). After application of UFs, the RfCs for all of the above inhalation effects were similar: $6 \times 10^{-2}$ to $8 \times 10^{-2}$ mg/m$^3$. This somewhat alleviates the concern that the availability of a neurotoxicity study in the TMB oral database would result in a substantially lower POD currently available for decreased monocytes.

EPA's *A Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002) recommends that the $UF_D$ take into consideration whether there is concern from the available toxicology database that the developing organism may be particularly susceptible to effects in specific organs/systems. TMBs (unspecified isomer) are able to cross the placenta (Cooper et al., 2001; Dowty et al., 1976); therefore, some degree of developmental toxicity is expected as it was observed following inhalation exposure. While general developmental toxicity is not expected to be a concern, as those types of endpoints (e.g., decreased fetal weight) were observed at concentrations much higher than effects observed in other systems, including the nervous system, there is also the possibility that exposure to TMB isomers may result in neurotoxicity in the developing organism. EPA's *Guidelines for Neurotoxicity Risk Assessment* (U.S. EPA, 1998) identifies specific effects observed in adult animals (e.g., cognitive and motor function) that can also affect the developing organism exposed in utero. The neurotoxicity guidelines (U.S. EPA, 1998) also indicate that neurotoxicants may have greater access to the nervous system in developing organisms due to an incomplete blood-brain barrier and immature metabolic detoxifying pathways. Lastly, EPA's *A Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002) also states that effects that may be mild or reversible in adults may produce more robust or permanent effects in offspring following developmental exposures. However, as discussed in Section 2.1.3, while the developing brain is potentially at greater risk of permanent injury due to exposure to neurotoxicants, there is uncertainty in the TMB and related compound database whether sufficient amounts of the toxic agent cross the placenta to elicit effects and whether the concentrations necessary to elicit effects are lower than those that result in neurotoxicity in the adult organism. There is evidence from perinatal inhalation toxicity studies in rats that low-level exposures to toluene (as low as 5 ppm) early in life (PNDs 4–12) may disrupt developmental processes that began early in embryogenesis (neuronal proliferation, synaptogenesis, myelination, etc.) and result in neurotoxicity (decrements in spatial learning) (Win-Shwe et al., 2010; Win-Shwe and Fujimaki, 2010). Therefore, concern still exists regarding the possibility that exposure to TMB may result in developmental neurotoxicity, possibly at levels lower than neurotoxicity in adult animals. In summary, a 3-fold $UF_D$ was applied to account primarily for the lack of both an adult and developmental neurotoxicity studies in the oral database for TMB isomers.

While the derivation of organ- or system-specific RfDs may be useful for subsequent cumulative risk assessments that consider the combined effect of multiple agents acting at a common site, only one adverse effect was identified as a critical effect in the available oral TMB database. Thus, only one chronic RfD was derived for TMBs via the application of the 300-fold composite UF (see above), based on increased monocytes following exposure to 1,3,5-TMB, as follows:

$$\mathbf{RfD = POD_{HED} \div UF = 3.01\ mg/kg\text{-}day \div 300 = 0.0101\ mg/kg\text{-}day = 1 \times 10^{-2}\ mg/kg\text{-}day}$$

**(rounded to one significant digit)**

While Adenuga et al. (2014) is a well-conducted study that evaluates a wide range of general toxicity endpoints in multiple organs/systems, the oral TMB database suffers from the lack of a neurotoxicity study. It is clear from the inhalation database for TMB that neurotoxicity is a critical endpoint for derivation of reference values. While Adenuga et al. (2014) (and the earlier Koch Industries (1995b) industry report) do report some clinical signs possibly indicative of neurotoxicity, they are too general to be used in the quantitative derivation of an RfD. However, the consistency with which neurotoxicity is observed in the TMB database, across all isomers following acute oral and acute, short-term, and subchronic inhalation exposures, is quite strong. Ultimately, the fact that oral and inhalation neurotoxic endpoints are qualitatively comparable, and that neurotoxic endpoints resulted in the most strongly supported RfCs in the inhalation database, it is reasonable to expect that neurotoxicity-based PODs would be important when calculating oral RfDs. Therefore, the available PBPK model for 1,2,4-TMB was used to perform a route-to-route extrapolation.

A route-to-route extrapolation from inhalation to oral for the purposes of deriving an RfD is possible using the existing inhalation data and the available 1,2,4-TMB PBPK model (Hissink et al., 2007), and was conducted to address the lack of suitable neurotoxicity data in the oral TMB database. The Hissink et al. (2007) model was chosen as an appropriate model because it was the only published 1,2,4-TMB model that included parameterization for both rats and humans, the model code was available, and the model adequately predicted experimental data in the dose range of interest. Using route-to-route extrapolation via application of PBPK models is supported by EPA guidance (U.S. EPA, 2002, 1994b) given enough data and the ability to interpret that data with regard to differential metabolism and toxicity between different routes of exposure. The available database for 1,2,4-TMB supports the use of route-to-route extrapolation; sufficient evidence exists that demonstrates similar qualitative profiles of metabolism (i.e., observation of dimethylbenzoic and hippuric acid metabolites) and patterns of parent compound distribution across exposure routes (Section C.2, Appendix C). Further, no evidence exists that would suggest that toxicity profiles would differ to a substantial degree between oral and inhalation exposures. In fact, in acute oral studies in rats (Tomas et al., 1999a; Tomas et al., 1999b), the observed neurotoxic effects of exposure to 1,2,4-TMB (i.e., alterations in motor function and electrocortical activity) are similar to effects observed following short-term exposures to 1,2,4-TMB via inhalation. This consistency of effect is also true for 1,3,5-TMB. Additional evidence of concordance of effects within the inhalation database is the similarity in types and magnitude of effects (i.e., decreased fetal weight) observed in rats exposed gestationally to either 1,2,4-TMB or 1,3,5-TMB (Saillenfait et al., 2005) (see Section 1.2.7 for a full description of the toxicological and toxicokinetic similarities among the TMB isomers). Lastly, it is reasonable to assume that had Adenuga et al. (2014) investigated more subtle markers of neurotoxicity, they would have been observed, since the PBPK model-predicted blood TMB levels were approximately 10-fold higher when estimated using the exposure paradigm in

Adenuga et al. (2014) compared to blood levels estimated from the inhalation exposures used in Korsak and Rydzyński (1996).

Therefore, assuming that oral exposure would result in the same systemic effect as inhalation exposure (i.e., altered CNS function, measured as decreased pain sensitivity (Korsak and Rydzyński, 1996)), an oral exposure component was added to the Hissink et al. (2007) PBPK model by EPA (Section C.2.3.5, Appendix C), assuming 100% absorption of the ingested 1,2,4-TMB by constant infusion of the oral dose into the liver. This is a common assumption when information about the oral absorption of the compound is unknown. The contribution of the first-pass metabolism in the liver for oral dosing was evaluated by simulating steady-state venous blood levels (at the end of 50 days of continuous exposure) for an average human at rest (70 kg) for a range of concentrations and doses; at low daily doses (0.1–10 mg/kg-day), equivalent inhalation concentrations result in steady-state blood concentrations 4-fold higher than those resulting from oral doses, indicating the presence of first-pass metabolism following oral exposure (see Figure C-18, Appendix C). This difference became insignificant for daily doses exceeding 50 mg/kg-day. To more accurately approximate patterns of human oral ingestion, ingestion was simulated as an idealized pattern of six events, each lasting 30 minutes. Twenty-five percent of the total daily dose was assumed to be ingested at each of three events beginning at 7 am, 12 pm (noon), and 6 pm (total of 75%). Ten percent of the daily dose was assumed to be ingested at events beginning at 10 am and 3 pm (total of 20%). The final 5% was assumed to be ingested in an event beginning at 10 pm. After the daily blood concentration profile achieved a repeating pattern, or periodicity, the weekly average blood concentration was then used to determine the HED.

The human PBPK model inhalation dose metric (weekly average blood concentration, mg/L) for the $POD_{ADJ}$ (0.099 mg/L) for decreased pain sensitivity was used as the target for the oral dose metric. The human PBPK model was run to determine what oral exposure would yield an equivalent weekly average blood concentration, and the resulting value of 3.5 mg/kg-day was then used as the $POD_{HED}$ for the RfD derivation, as follows:

**$RfD = POD_{HED} \div UF = 3.5$ mg/kg-day $\div 300 = 0.0117$ mg/kg-day $= 1 \times 10^{-2}$ mg/kg-day (rounded to one significant digit)**

This calculated value of $1 \times 10^{-2}$ mg/kg-day is equal to the RfD calculated from the oral 1,3,5-TMB data for increased monocytes. This is consistent with what was observed in the inhalation database. The RfCs for hematotoxicity endpoints (decreased segmented neutrophils for 1,2,3-TMB [$6 \times 10^{-2}$ mg/m$^3$] and decreased clotting time for 1,2,4-TMB [$8 \times 10^{-2}$ mg/m$^3$]) were identical or similar to the value estimated for decreased pain sensitivity following exposure to 1,2,4-TMB or 1,2,3-TMB ($5 \times 10^{-2}$ to $6 \times 10^{-2}$ mg/m$^3$). This indicates that some endpoints in the hematological system are equally as sensitive to exposure to TMB isomers as endpoints in the nervous system. Further supporting this conclusion is the fact that a route-to-route extrapolation

of the most sensitive hematological effect following inhalation exposure to 1,2,4-TMB (decreased clotting time, $POD_{ADJ}$ = 0.1335 mg/L) results in a $POD_{HED}$ of 4.3 mg/kg-day and a final RfD of $1 \times 10^{-2}$ mg/kg-day (after application of the composite 300-fold UF). Confidence in the route-to-route derived RfD is additionally strengthened given that the PBPK model used is a well-characterized model determined to be appropriate for this assessment. Ultimately choosing the route-to-route neurotoxicity-based RfD (based on 1,2,4-TMB) as the RfD for all TMB isomers is supported by multiple lines of evidence in the oral and inhalation database. First, all three isomers are observed to elicit similar neurotoxic effects in rats in acute oral and inhalation studies and short-term and subchronic inhalation studies. For example, following inhalation exposure, while 1,2,3-TMB is observed to decrease pain sensitivity at lower concentrations than 1,2,4-TMB (LOAEL values of 123 versus 492 mg/m$^3$, respectively), the magnitude of decreased pain sensitivity is similar for 1,2,4-TMB and 1,2,3-TMB, especially at the low- and mid-concentrations. This similarity of effect in the low-dose region of the dose-response curve is exhibited by identical RfC values derived from isomer-specific data: $6 \times 10^{-2}$ mg/m$^3$. Similarities in blood:air and tissue:air partition coefficients and absorption into the bloodstream between TMB isomers support the conclusion that internal blood dose metrics for 1,3,5-TMB and 1,2,3-TMB would be similar to those calculated for 1,2,4-TMB using the available PBPK model. Also, the qualitative metabolic profiles for the two isomers are similar, with dimethylhippuric acids being the major terminal metabolite for both isomers, so that first-pass metabolism through the liver is not expected to differ greatly between the three isomers. **Based on these considerations, and the confidence in the route-to-route extrapolation, the RfD for TMBs is $1 \times 10^{-2}$ mg/kg-day, based on decreased pain sensitivity observed in rats exposed to 1,2,4-TMB.**

### 2.2.4. Uncertainties in the Derivation of the Reference Dose for TMBs

As the oral RfD for TMBs was based on a route-to-route extrapolation in order to determine the oral dose that would result in the same effect (i.e., decreased pain sensitivity) as inhalation exposure in Korsak and Rydzyński (1996), many of the uncertainties regarding this derivation are the same as those for the RfC for TMBs (see Section 2.1.6), with the exception of the uncertainty surrounding the route-to-route extrapolation. The model used to perform this route-to-route extrapolation is a well-characterized model considered appropriate for the purposes of this assessment. One source of uncertainty regarding the route-to-route extrapolation is the assumption of 100% bioavailability (i.e., 100% of the ingested 1,2,4-TMB would be absorbed and passed through the liver). If not all of the compound is bioavailable, a lower blood concentration would be expected compared to the current estimate, and thus, a higher RfD would be calculated. Although there is uncertainty surrounding the assumption of 100% bioavailability, this is a common assumption when data on oral absorption are unknown. Further, the use of this assumption results in an RfD value that is lower than one that would be derived using a bioavailability less than 100%. Therefore, even in the consideration of the uncertainty surrounding this decision, the assumption of 100% bioavailability results in a health- protective RfD. There is

additionally some uncertainty about adopting the RfD based on the route-to-route extrapolation of decreased pain sensitivity in rats exposed to 1,2,4-TMB for the RfD for both 1,3,5-TMB and 1,2,3-TMB. However, as discussed in Section 2.1.6, all three TMB isomers share multiple commonalities and similarities regarding their chemical, toxicokinetic, and toxicological properties that support adopting one isomer's value for the other.

### 2.2.5. Confidence Statement for the Reference Dose for TMB

The confidence in the oral database for TMB is low as it only contains acute oral studies investigating neurotoxicity endpoints for multiple isomers, and one subchronic study investigating general toxicity endpoints for one isomer (1,3,5-TMB). This database was used to derive an RfD, but given the concern over the lack of a suitable neurotoxicity study, the confidence in this RfD is low. A PBPK model was utilized to perform a route-to-route extrapolation to determine a POD for the derivation of the RfD from the Korsak and Rydzyński (1996) inhalation study and corresponding critical effect. The confidence in the study from which the critical effect was identified, Korsak and Rydzyński (1996), is low to medium (see Section 2.1.7). The inhalation database for 1,2,4-TMB includes acute, short-term, subchronic, and developmental toxicity studies in rats and mice. However, confidence in the overall database for TMB is low to medium because it lacks chronic and developmental neurotoxicity studies, and the studies supporting the critical effect predominantly come from the same research institute. Reflecting the confidence in the study and the database and the uncertainty surrounding the application of the available PBPK model for the purposes of a route-to-route extrapolation, **the overall confidence in the RfD for TMB is low**.

### 2.2.6. Calculation of Subchronic Reference Doses for TMBs

In addition to providing RfDs for effects in the hematological and nervous systems, this document also provides subchronic RfDs for less-than-lifetime exposures. In the case of TMBs, the oral 1,3,5-TMB study and 1,2,4-TMB inhalation study used for the route-to-route extrapolation to calculate the chronic RfDs were both subchronic duration. Therefore, the methods used to calculate subchronic RfDs is identical to that used for calculation of chronic RfCs, minus the application of a $UF_S$. This results in a composite UF of 100 ($UF_A$ of 3, $UF_H$ of 10, $UF_S$ of 1, and $UF_D$ of 3). Dividing the POD for hematological effects (3.03 mg/kg-day) and neurotoxicity effects (3.5 mg/kg-day) by the composite UF of 100 results in subchronic RfDs of $3 \times 10^{-2}$ or $4 \times 10^{-2}$ mg/kg-day for decreased monocytes and decreased pain sensitivity, respectively. The subchronic oral RfD is intended for use with exposures for more than 30 days, up to approximately 10% of the lifespan in humans. **The subchronic RfD was set to $4 \times 10^{-2}$ mg/kg-day based on neurological effects following exposure to 1,2,4-TMB**.

## 2.3. CANCER RISK ESTIMATES FOR TMBs

Under the U.S. EPA *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005), the database for 1,2,4-TMB, 1,2,3-TMB, and 1,3,5-TMB provides "inadequate information to assess carcinogenic potential." Information available on which to base a quantitative cancer assessment is lacking, and thus, **no cancer risk estimates for either oral or inhalation exposure are derived.**

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