FDA's Response to Comments from the External Peer Review of the Expanded Decision Tree: Ranking Toxic Potential (March-September 2024)

06/12/2025

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I. INTRODUCTION

The Food and Drug Administration (FDA) is a scientific regulatory agency responsible for ensuring the safety of the nation's domestically produced and imported foods, cosmetics, drugs, biologics, medical devices, radiological products, and tobacco products. The FDA supports the development and evaluation of new toxicological methods and technologies and integrating them into risk and safety assessments as appropriate. One area of focus is enhancing the FDA's toxicology predictive capabilities to potentially reduce reliance on animal testing. A notable tool developed by the FDA is the Expanded Decision Tree (EDT).

The EDT applies a series of chemical structure-based questions to classify chemicals with diverse structures into one of six classes based on chronic oral toxic potential, ranging from Class I (exhibiting very low toxic potential) to Class VI (exhibiting the highest toxic potential). Each class has a threshold of toxicological concern (TTC) level calculated from existing toxicity studies for related compounds. According to the TTC concept, if oral exposure to a compound doesn't exceed its class's TTC level, the compound is predicted to present a low probability of risk.

The FDA developed the EDT to build upon the knowledge and approaches developed by Cramer et al in 1978. The EDT incorporates new scientific studies and mechanistic understanding and increases the number of substances in the knowledgebase substantially. The FDA conducted an external, independent peer review of the EDT to evaluate the applicability, limitations, relevance, reliability, reproducibility, and sensitivity to ensure it is fit for use. This review aimed to evaluate, analyze, and verify that the EDT is scientifically sound and suitable for screening and prioritization of chemicals in food that lack compound specific safety data and sufficiently predicts conservative safe levels of intake (i.e., TTCs) to inform food chemical safety assessment.

Goldbelt, LLC, an independent contractor, coordinated an external peer review of the EDT for the FDA. For this review, Goldbelt selected four experts to evaluate whether the FDA has clearly defined its guidelines and definitions and if the EDT questions effectively capture the necessary structural features to accurately classify chemicals based on their chronic oral toxic potential. The experts were tasked with assessing the sufficiency and correctness of the example structures provided after each EDT question, evaluating the adequacy of the practice set, and identifying any missing metabolic precursors or unaddressed congeneric groups of chemicals. They also reviewed the comprehensiveness of the explanations for the pre-validation EDT questions. Additionally, the reviewers examined the adequacy of the validation process and study selection criteria for both databases (DBs)—the pre-validation (original) and the validation DBs. They evaluated the appropriateness of the duration adjustment factors used to derive chronic no-effect levels from non-chronic studies, as well as the coverage of the combined toxicological DB. Finally, the reviewers were asked to provide any additional comments or suggestions for improvement.

In section II of this report, we list the charge questions given to reviewers. Section III

provides a summary of the comments from the peer reviewers organized question by question, the FDA's responses to these comments, and a description of the changes made to the peer review document in response. Finally, the individual peer reviewer comments are included in the Appendix.

Below are the names and affiliation of the peer reviewers:

Prof. Mark Cronin, Ph.D.
Liverpool John Moores University, UK

Kim Li, Ph.D., DABT
Kim L Li, LLC, California, USA

Mark Nelms. Ph.D.
RTI International

George Kass, Ph.D., ERT
European Food Safety Authority (EFSA)
University of Surrey, UK

II. CHARGE TO REVIEWERS

Phase I Peer Review Charge Questions:

Questions for section 1 (The Expanded Decision Tree (EDT)):

Question 1: Has FDA clearly explained, with adequate examples, all guidelines and definitions for use with the EDT? If not, please provide suggestions for alternate text and/or additional examples.

Question 2: Are all EDT questions clear as to which structural features they are describing? If no, please identify the question by its number, explain why you find the question ambiguous or confusing, and suggest alternative text to ensure that it is clear what kind of structural features the question is aiming to capture.

Question 3: Most questions place compounds into one of six classes of toxic potential depending on their structural features. Does the EDT place the type of compounds that are captured at each question into the appropriate class of toxic potential? If not, please explain why and provide a recommendation for the appropriate class of toxic potential.

Question 4: Commonly, structurally related compounds (e.g., γ-diketones) can have common toxicological endpoint (in this case γ-diketone type neurotoxicity). Compounds that can either hydrolyze and/or metabolize to these compounds can exhibit the same type of toxicity. FDA aimed to capture hydrolytic and metabolic precursors of structurally related compounds with similar toxicities at numerous questions. Are there any questions where

you can suggest any possible metabolic and/or hydrolytic precursors to the types of compounds addressed questions that are currently not mentioned/captured in the question?

Question 5: Are the example structures provided after each EDT question correct and adequate for understanding what type of compounds the question aims to capture? Are there different or additional example structures for any of the questions that would help increase the understandability of the question?

Question 6: Are there any congeneric groups that the EDT does not adequately address, but for which enough safety data exist that could serve as the basis of additional EDT questions to address these groups? If yes, please identify and provide all toxicological data for the congeneric group(s) that may form the basis of one or more additional questions. If possible, please propose the wording for such additional EDT questions. (Substances within a congeneric group are structurally and metabolically similar.)

Question 7: Should any questions be further subdivided to ensure a more refined grouping of related substances? If yes, please suggest wording for the refined question(s) and provide the data justifying the suggestion.

Question 8: Are there any terms used in the EDT questions that should be added to the guidelines and definitions section to help users of the EDT? If yes, what additional terms should we define?

Questions for section 2 (The Expanded Decision Tree Chemistry, Toxicity, and Metabolism Database (EDT DB)):

Question 9: Has FDA clearly explained where the toxicological data found in the EDT DB were collected from? If not, what additional information should we provide?

Question 10: Has FDA clearly explained the study selection criteria and provided adequate information and/or data to support its opinion that these criteria are appropriate for data inclusion in the DB? If not, what additional information should we provide?

Question 11: FDA used various factors based on study duration to derive duration adjusted no-effect-levels (NELs) to estimate chronic NELs. Has FDA provided adequate information and/or data to support its opinion that these duration adjustment factors are adequate to derive chronic NELs? If you generally agree, are there any exceptions in which these factors might be problematic to the derivation of duration adjusted NELs?

Questions for section 3 (The Preliminary (Pre-validation) Threshold of Toxicological Concern Levels):

Question 12: Based on Figure 2 and all other information provided, in your opinion, does the EDT better resolve the differing toxic potentials of chemicals with broad structural variation compared to the CDT? Please explain why or why not.

Questions for section 4 (The Validation of the Expanded decision Tree):

Question 13: Has FDA clearly explained the source of the validation DB and how the data was verified pre-validation? If not, what additional information should we provide?

Question 14: Has FDA clearly laid out how the validation DB received from EPA was processed to enable its use for the external validation of the EDT? If not, please explain why not.

Question 15: Has FDA provided adequate information and/or data to show that the validation DB was processed appropriately for its intended use? If not, what additional information should we provide?

Phase II Peer Review Charge Questions:

Question 16: Some of the pre-validation EDT questions were updated, and some new sub- and sub-sub-questions were created based on the validation results. Has FDA provided adequate information to justify all updates? If not, which changes/updates were not fully justified and what information should we provide to justify them?

Question 17: Was the validation adequate to show that the EDT is suitable for the classification of compounds in its applicability domain according to their toxic potentials? If not, describe what type of validation would be needed.

Question for section 5 (Conclusions):

Question 18: Has FDA provided adequate information and/or data to support the conclusions found in this section? If not, what additional information should we provide?

Appendix 1 aims at providing a brief explanation of each EDT question. By no means are these explanations meant to be comprehensive. With that in mind, please respond to the following questions.

Question 19: Are all explanations clear and concise? If not, please identify the explanation by question number and elaborate as to how we can more clearly explain the question.

Question 20: Should FDA add anything to these explanations to improve the reader's understanding of each question's rationale? If yes, please identify the explanation by question number and explain how we should revise. Please note that these explanations were designed to be concise and not all-encompassing.

Appendix 2[1] contains the combined, finalized EDT Chemistry, Toxicology and Metabolism DB on which the finalized TTCs were based.

Question 21: Are the set of chemicals in the database sufficient to cover the chemical domain of applicability described in the document? If not, please explain.

[1] Appendix 2 was incorrectly noted as Appendix 3 in the original questions.

Overall question:

Question 22: Do you have any other comments or suggestions?

III. SUMMARY OF PEER REVIEWER COMMENTS AND FDA RESPONSE

Phase I:

Question 1: Has FDA clearly explained, with adequate examples, all guidelines and definitions for use with the EDT? If not, please provide suggestions for alternate text and/or additional examples.

Summary of general impressions:

The feedback on section 1.5 (guidelines and definitions) of the EDT peer review document reveals that while the section is generally well-received and informative, there are specific areas where improvements could enhance clarity and utility. While the definitions and guidelines provided are mostly clear, reviewers suggested refinements to improve understanding and alignment with general scientific concepts.

Key areas for improvement include:

1. **Reorganization and Clarity:** Several peer reviewers recommended reorganizing the definitions and examples to improve logical flow. For example, rearranging the order of structural features and providing clearer definitions for terms like "alicyclic," "heteroaromatic," and other terms would enhance comprehension. There was also a suggestion to include structures for fundamental classes of compounds, such as aliphatic and aromatic rings, which would be especially useful if the scheme is implemented computationally.
2. **Definitions and Examples:** Some definitions, particularly those specific to the EDT, were noted as differing from general literature, creating potential confusion. Suggestions included defining terms like "reduction" more explicitly and clarifying the concept of "reactive moiety" by specifying whether it refers to electrophilic or nucleophilic groups, and/or something else. Adding references to figures or diagrams, such as those illustrating SMART patterns[2] or functional groups, could also improve understanding.
3. **Consistency and Detail:** There were comments on inconsistencies and missing details, such as the treatment of organic salts and metal ion salts in their neutral forms, which may not fully align with general reactivity and toxicity concepts. Some terms like "Bay and Fjord regions" were noted as needing clearer definitions or descriptions.

[2] SMART patterns are used to identify key structural features in molecules that can predict their reactivity, stability, or behavior under various conditions. In chemistry, these patterns help in understanding how functional groups and molecular structures influence chemical properties. In toxicology, SMART patterns are applied to predict the potential toxicity of chemicals based on their structural motifs and known toxicological profiles.

4. **Section Organization:** The comments suggested that section 1.5 could be more effectively organized. This could involve presenting information in a table format and clearly stating the section's purpose and expectations. Providing a clear introductory statement about the need for a background in organic chemistry could also help users better navigate the section.
5. **Applicability Domain:** There was a call for a section describing the applicability domain of the EDT scheme, such as molecular weight limits, to provide context for its use.

Overall, the feedback highlights a need for greater clarity and organization in section 1.5 to ensure it is accessible to a wide range of users and aligns with both general scientific understanding and practical application.

FDA response:

We appreciate the reviewers' responses and suggestions for enhancing the clarity, accuracy, precision, and comprehensiveness of section 1.5 ("Guidelines and Definitions for Use with the EDT"). Although organizing this section into a table may be impractical due to the presence of large figures designed to aid user understanding, we have made the following updates to section 1.5 based on the peer reviewers' comments to improve it:

1. **Applicability Domain**: Added a section defining the chemical applicability domain of the EDT and clarified that the tool is designed to predict the toxic potential of compounds through *oral* exposure. The newly added section states "Applicability domain of the EDT: all compounds except unhydrolyzable polymers, proteins, elements, inorganic substances, and substances with undefined structures. Please note that ingested particles may have varying bioavailability and toxicity depending on their size. The EDT is not designed to estimate safe intake levels (i.e., TTCs) based on particle size and should only be applied to substances within its applicability domain. While there is no cutoff for molecular weight (MW) when applying the EDT, the MW range of substances in the combined EDT DB is 30.03-2285.61 Da. Some of the hydrolyzable polymers within the structural applicability domain of the EDT may have MWs that exceed this range. In case of hydrolyzable polymers, the EDT assumes complete hydrolysis to monomeric units. Additionally, please note that the EDT is designed specifically to sort compounds based on/according to their relative chronic toxic potential through oral exposure only."
2. **Section Renaming**: Renamed section 1.5 from "Guidelines and Definitions for Use with the EDT" to "Applicability Domain and Definitions for Using the EDT" to better reflect its content.
3. **Explanation of Term Modifications**: Added a brief explanation for modifications made to common chemistry terms to simplify the EDT language to sections 1.5 and 4.5.2 (sections providing the definitions for the pre- and post-validation EDT, respectively): "Although most common chemistry terms in the EDT are used as they are in scientific literature, some terms have been modified to simplify the language of the EDT questions."
4. **Reorganized Definitions**: Reordered the definitions for improved logical flow. The

revised section now begins with the applicability domain of the EDT, followed by terms related to major structural features and descriptors, positions, regions, functional groups, reactions (hydrolysis, reduction, and enolization), and finally, the equivalency of $-CF_3$ to a single halogen.

5. **Example Structures**: Added example structures for categories E-J (formerly A-F), including aliphatic, acyclic, alicyclic, heterocyclic, heteroaromatic, and aromatic/aryl. Included examples and explanations for related terms within each definition, such as alkane, alkene, polyalkene, etc., within the definition of aliphatic.
6. **Updated Definition of Alicyclic**: Refined the definition from “Alicyclic means the presence of a ring composed of only carbon atoms with or without the presence of ring alkene(s) without forming an aromatic ring” to “Alicyclic refers to a molecule where all rings are composed solely of carbon atoms. These rings may contain ring alkenes but do not form an aromatic ring.”
7. **Updated Definition of Heterocyclic**: Clarified the definition from “Heterocyclic means the presence of a ring with at least one ring atom other than carbon (commonly N, O, and/or S)” to “Heterocyclic refers to a molecule that contains at least one ring structure where at least one of the ring atoms is not carbon, commonly nitrogen (N), oxygen (O), or sulfur (S).”
8. **Refined Definition of Heteroaromatic**: Improved clarity and grammar in the definition from “Heteroaromatic means that the substance contains at least one ring containing at least one ring heteroatom (commonly N, O, and/or S) that has a completed cyclic array of [4n+2]π electrons (e.g., furan, pyrrole, 1,3-imidazole, thiazole, and pyridine). Heteroaromatic substances are a subgroup of heterocyclic substances.” to “Heteroaromatic refers to a substance that contains at least one ring with at least one ring heteroatom (commonly N, O, or S) and a fully conjugated cyclic array of [4n+2]π electrons (e.g., furan, pyrrole, 1,3-imidazole, thiazole, and pyridine). Heteroaromatic compounds are a specific subgroup of heterocyclic compounds.”
9. **Refined Definition for Solo, Duo, Trio, Quartet**: Clarified the definition from “Solo, duo, trio, quartet: in polycyclic aromatic hydrocarbons (PAHs), a solo, duo, trio, and quartet contains one, two, three, or four adjacent carbons, respectively, each of which can be bonded to an atom other than an atom from an aromatic ring” to “Solo, duo, trio, quartet: In polycyclic aromatic hydrocarbons (PAHs), the terms solo, duo, trio, and quartet refer to configurations where one, two, three, or four adjacent carbon atoms, respectively, are bonded to atoms other than those within the aromatic ring. For example, in the provided structures, each of the three ‘trio’ carbons are bonded to a hydrogen atom. That is, these trio carbons are bonded to hydrogen atoms outside the aromatic ring structure.”
10. **Removed Confusing Explanations**: Removed the explanation regarding the treatment of organic and metal ion salts from the definition section due to causing confusion and redundancy, as this is addressed in specific EDT questions. Furthermore, the treatment of organic and metal ions varied from EDT question to question and, as such, should not have been addressed together. FDA notes that the EDT software, which is currently under development and will be made publicly available, will automatically handle the treatment of salts. Users will not need to address this issue manually.
11. **Removed Definition for Reactive Moiety**: Eliminated the definition for "reactive

moiety" as it was ambiguous and unnecessary for interpreting EDT questions.
12. **Added Definition for Reduction**: Included a new definition for “reduction.” (i.e., Reduction is a chemical reaction where a species undergoes a gain of electrons or a decrease in its oxidation state. This process can involve the addition of hydrogen atoms or the removal of oxygen atoms from a molecule. Reduction is typically associated with the transfer of electrons from another substance that is being oxidized.)
13. **Added Definitions for Fjord and Bay Regions**: Added new definitions for “fjord” and “bay” regions in polycyclic aromatic hydrocarbons. (i.e., Bay and Fjord regions: The bay region is characterized by the presence of a "bay" or "indentation" in the aromatic system. The fjord region refers to a structural feature in a molecule where there is a pronounced "fjord" or "trough" between two aromatic rings.)
14. **Corrected Bolding of Defined Terms**: Ensured all defined terms, including “corresponding,” are properly bolded in the EDT questions.
15. **Updated Figures**: Reordered the example structures for bridged, fused, spiro fused, and singly bonded rings to match their order in the definition section and labeled “bridgehead atoms” for clarity.

These changes were also applied to the post-validation EDT (section 4.5.2).

FDA notes that in response to peer review question 8, one of the peer reviewers suggested adding definitions for dimer, connector, conjugated, and organyl. Please see reviewer’s comment and FDA’s response to this comment at question 8. In summary, FDA added definitions for these terms to section 1.5.

Question 2: Are all EDT questions clear as to which structural features they are describing? If no, please identify the question by its number, explain why you find the question ambiguous or confusing, and suggest alternative text to ensure that it is clear what kind of structural features the question is aiming to capture.

Summary of general impressions:

The peer reviewers generally found the EDT questions to be mostly clear regarding the structural features they are intended to describe, with some noted areas for improvement:

1. **Clarity and Structure**: Most reviewers agreed that the questions are clear, but some suggested improvements in formatting and organization to enhance readability and understanding. For example, suggestions included using new lines for sub-sub-questions, ensuring consistent bolding of defined terms, and clearly identifying where answers should lead within each question.
2. **Definitions and Terminology**: Some reviewers noted that while the definitions are generally clear, there are specific terms and structures that could be more precisely defined or illustrated. For example, terms like "corresponding" and "azide," as well as the usage of symbols and functional groups, could benefit from clearer explanations or examples.
3. **Structural Features and Examples**: There were comments on the need for additional examples or clarifications for certain questions, such as including illustrations of

specific functional groups, clarifying the role of substituents, and ensuring that the definitions align with the intended structural features.

4. **Coverage and Interlinkages**: Concerns were raised about the completeness of the questions, particularly regarding naturally occurring compounds and how they are addressed compared to man-made compounds. There was also a mention of potential inconsistencies in how different questions might lead to varying class assignments for the same chemical.

Overall, the reviewers acknowledged the strengths of the EDT questions but highlighted areas where additional clarity and refinement could improve their effectiveness.

FDA response:

We appreciate the reviewers' feedback and suggestions for enhancing the clarity, accuracy, precision, and comprehensiveness of the EDT questions. Based on their comments, we have made the following updates:

1. **Improved Question Organization:**
   a. **Visual Separation:** Added lines (**----------**) after each main question to clearly indicate the end of one question and the start of the next.
   b. **Color Coding:** Changed formatting to improve visibility. FDA notes that while the colors are not required to distinguish main, sub-, and sub-sub-questions, they improve visibility. The three colors chosen (i.e., strong medium blue — Hex: #0072B2, dark orange — Hex: #B35C00, and green — Hex: #00704A) are generally distinguishable by people with all types of color vision, including the most common forms of color blindness, and provide adequate contrast against each other and the background.):
      i) Main question numbers are **now bolded and in "dark" orange**.
      ii) Letters denoting sub-questions are **bolded and in "strong medium" blue**.
      iii) Sub-sub-questions markers (i, ii, iii, etc.) are **bolded and in green**.
   c. **Reorganization:** Reformatted sub-questions with many sub-sub-questions so that each sub-sub-question starts on a new line for better readability.
2. **Clarified Symbols and Terms:** In some questions, the symbol "=" stood for both double bonds and equality. To avoid confusion, FDA changed "=" to "is" where it denotes equality (e.g., X is C, N, O, or S). Now "=" only represents double bond.
3. **Bolded Defined Terms:** Based on the same comment for question 1, FDA ensured all defined terms are properly bolded in the EDT questions.
4. **Highlighted Structural Features:** Added specific examples of structural features described in the questions where feasible (e.g., Q6f i) and ii)). Note that the post-validation EDT has more examples compared to the pre-validation version reviewed during this phase (i.e., Phase I).
5. **Clarified Logical Connectives:** Added missing logical connectors ("or", "and", "and/or") between sub-questions to clarify the intended relationships.
6. **Reworded Questions for Clarity:** Reworded Q1e) and other questions (e.g., Q47f)) for improved clarity. For instance, Q47f): "In addition, all rings other than the tetrahydropyran ring that is fully substituted, all rings should have a minimum of two

substitutions." was changed to "Additionally, except for the fully substituted tetrahydropyran ring, every other ring should have at least two substitutions."

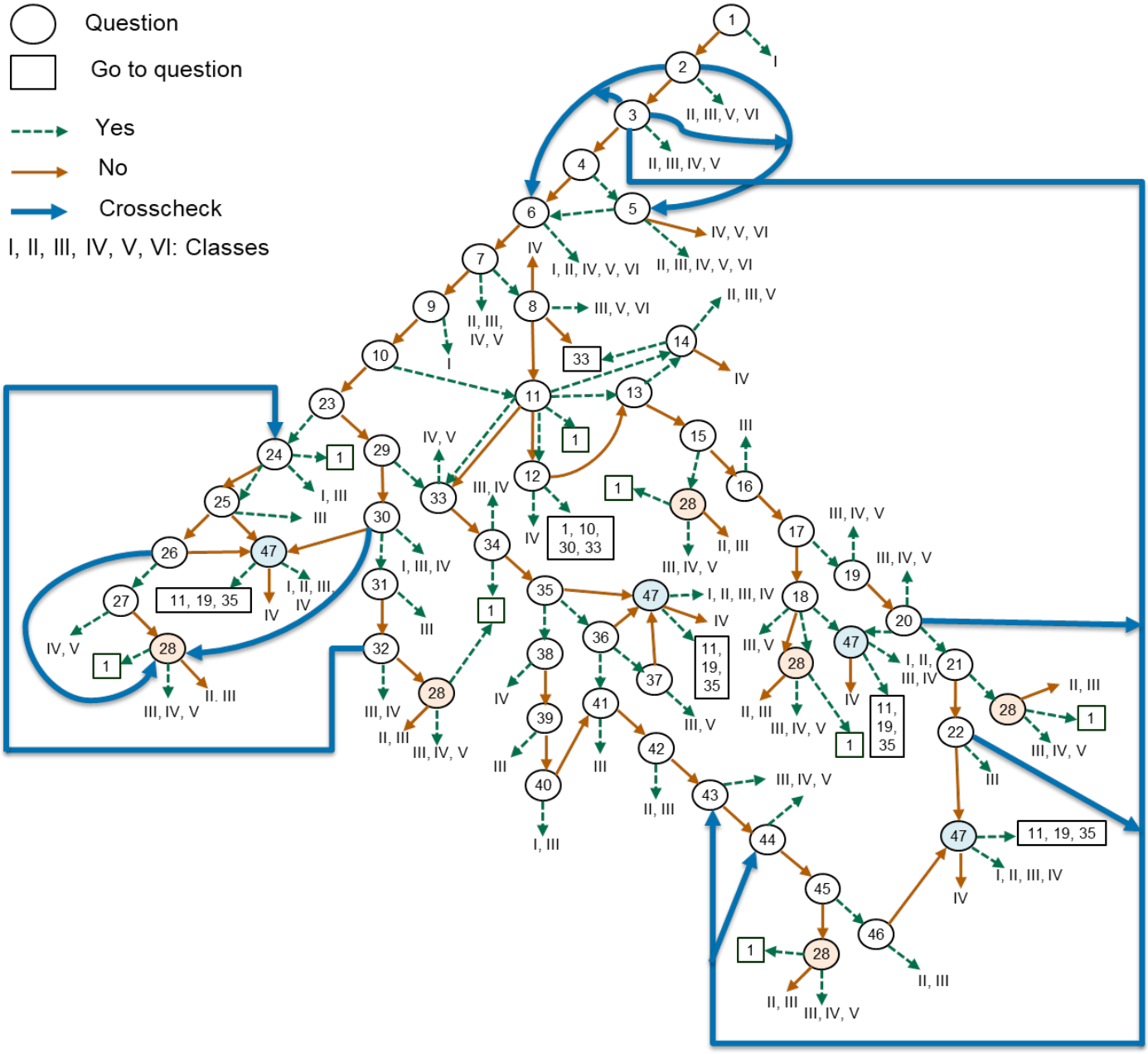
7. **Corrected Technical Details:**
   a) Fixed the SMARTS pattern error in Q3f) to accurately reflect the intended structure (i.e., changed from N^{-}-N^{+}≡N to N^{-}-N^{+}≡N).
   b) Clarified a term in Q6c) (i.e., what "additional" refers to) and symbol in Q12e) (i.e., 4≤ was changed to ≤4).
8. **Additional Changes:** Added missing "If yes" in Q18 ii) for completeness.

These updates have been applied to the post-validation EDT questions wherever applicable.

Some peer reviewers observed that the pre-validation EDT questions covered a limited range of natural toxins. The FDA has since updated the post-validation EDT to include a broader spectrum of natural toxins. The peer reviewers did not have access to the post-validation EDT at the time of the above comments.

FDA's response to individual peer review comments on question 2 not addressed above:

1. **Bisphenol A Classification:** One reviewer asked about the classification process for bisphenol A following EDT question (Q) 43. The FDA clarifies that if the response to Q43 is "no," users are directed to Q44. If the response to Q44 is also "no," users are directed to proceed to Q45. A "no" response at Q45 leads to Q28, where the final classification of the compound is determined.
2. **Interlinking Questions:** One peer reviewer mentioned, "It is difficult to reconstruct how the different questions are interlinked. Therefore, the fact that two different questions can address the same chemical and result in different class assignments may or may not occur when using the EDT. For example, TCDD can be classified in Q8b) as Class VI, but also in Q18a) as Class V." The FDA clarifies that once TCDD is classified in Q8b), it does not proceed to Q18a). Q8b) is specifically designed to classify highly toxic compounds like TCDD directly into Class VI. Structurally similar compounds with lower toxic potential, which are not classified at Q8b), are directed to other questions and may be classified at Q18a) into Class V.
3. **Overview of EDT Flow:** To assist users in understanding the flow and interlinking of questions within the EDT, the FDA has included the figure below in the post-validation EDT peer review document (see section 4.5.4 ("The post-validation EDT schema")) that outlines these relationships (for users of assistive technology or readers requiring a text-based version, see Appendix A for a description of Schema 1.):



Schema 1. The flow and interlinking of questions within the EDT

Question 3: Most questions place compounds into one of six classes of toxic potential depending on their structural features. Does the EDT place the type of compounds that are captured at each question into the appropriate class of toxic potential? If not, please explain why and provide a recommendation for the appropriate class of toxic potential.

Summary of general impressions:

The peer reviewers generally found that the EDT places compounds into appropriate

classes of toxic potential based on structural features, aligning with the expected levels of toxicity. They acknowledged the FDA's strong effort in classifying chemical classes correctly, though they noted that some refinements might be needed with increased use of the tree.

Key points and suggestions from the reviewers include:

1. **Overall Classification**: Most reviewers agreed that the EDT correctly classifies compounds into the appropriate toxicity classes, although they noted that the scheme is still evolving. They suggested that future refinements might be necessary based on practical use.
2. **Complexity and Usability**: One reviewer found section 1.7 (the technical description of chemistry) difficult to understand and suggested simplifying the presentation or using tables to clarify which chemistries correspond to each EDT class. Another reviewer proposed comparing the EDT classes directly with the Cramer scheme to aid understanding.
3. **Documentation and Clarity**: There was a call for clearer documentation, including:
   a) The peer reviewers suggested providing a concise overview of the chemical analysis, which could be presented in a publication or integrated into a software application to make it more accessible.
   b) Creating a table that directly links different types of chemistry to their corresponding EDT classes for easier reference.
   c) Reviewers noted that integrating the explanations from Appendix 1 more seamlessly with the main questions would reduce the need for frequent back-and-forth navigation, making it easier to follow the information.
4. **Endogenous Compounds and Exceptions**: Some reviewers identified specific issues with classifying certain compounds, such as endogenous phosphorylated compounds (e.g., phytic acid) and TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin). They noted inconsistencies for TCDD and potential overclassification for some phosphorylated compounds.
5. **Mechanistic Information**: There was interest in linking the EDT questions to mechanistic information and relevant Adverse Outcome Pathways (AOPs), though the feasibility of this approach was questioned.
6. **Decision Tree Approach**: One reviewer raised a fundamental concern about the decision tree approach, suggesting that it might not always be the most appropriate method. They proposed evaluating all alerts and using the most conservative classification for better protection and transparency.

Overall, the feedback highlights a strong initial foundation for the EDT while pointing out areas for improvement in clarity, documentation, and the potential for refinement based on practical use and further research.

FDA response:

We appreciate the reviewers' feedback on whether the EDT places the type of compounds that are captured at each question into the appropriate class of toxic potential.

In response to the peer reviewers' feedback, the FDA notes that it plans to periodically update the EDT to ensure it aligns with the latest scientific understanding of modes of toxic action and integrates new safety data as it becomes available.

Regarding the chemistries associated with each EDT class, the FDA notes that the chemistries within each class are highly varied and encompass compounds with broad structural diversity. Compounds with markedly different structures, metabolic pathways, toxicity endpoints, and modes of toxic action may be assigned the same EDT class. For instance, Class I includes a wide range of compounds with very low toxic potential, such as aliphatic linear and methyl-substituted primary alcohols, certain dicarboxylic acids, amino acids, various lactones, sugars, sugar alcohols and acids, bile acids, benzoic acid and related compounds, among numerous other chemical classes.

Moreover, even within the same chemical class, compounds can be categorized into different EDT classes. For example, linear or simply branched aliphatic acyclic hydrocarbons can be placed into different classes based on their specific structures. While hexane is classified under Class IV at Q28d(i)), substances with a terminal double bond conjugated with another double bond (i.e., terminal dienes) may be classified into either Class IV or III at Q28s(i)) or Q28s(ii)), respectively. Other compounds within this chemical class may be categorized into Class I at Q9.

Furthermore, assigning compounds to a single representative chemical class can be fraught with difficulties. While structurally simple compounds such as ethanol, acetaldehyde, or acetic acid can be easily assigned to primary alcohols, aldehydes, and carboxylic acids, respectively, assigning compounds with more than one functional group and/or complex skeleton to a single representative chemical class, for the purposes of describing the compounds sorted into the various EDT classes, can be extremely difficult as their toxic potential and mode of toxic action are a result of their various structural features and are often not due to a single structural feature. For example, the chemical classifications of 1) disodium;4-[4-[[5-(2-bromoprop-2-enoylamino)-2-sulfonatophenyl]diazenyl]-3-methyl-5-oxo-4H-pyrazol-1-yl]-2,5-dichlorobenzenesulfonate (Lanasol Yellow 4G, CAS 70247-70-0), 2) ethyl 2-[[(1R)-1-cyclohexyl-2-[(2S)-2-[[4-[(Z)-N'-hydroxycarbamimidoyl]phenyl]methylcarbamoyl]azetidin-1-yl]-2-oxoethyl]amino]acetate (Exanta, CAS 192939-46-1), and 3) methyl 3-chloro-5-[(4,6-dimethoxypyrimidin-2-yl)carbamoylsulfamoyl]-1-methylpyrazole-4-carboxylate (Halosulfuron-methyl, CAS 100784-20-1) into a single chemical class is not possible due to their structural complexities (see Figures A, B, and C below where the potential major chemical classifications and the corresponding structural features are color coded).

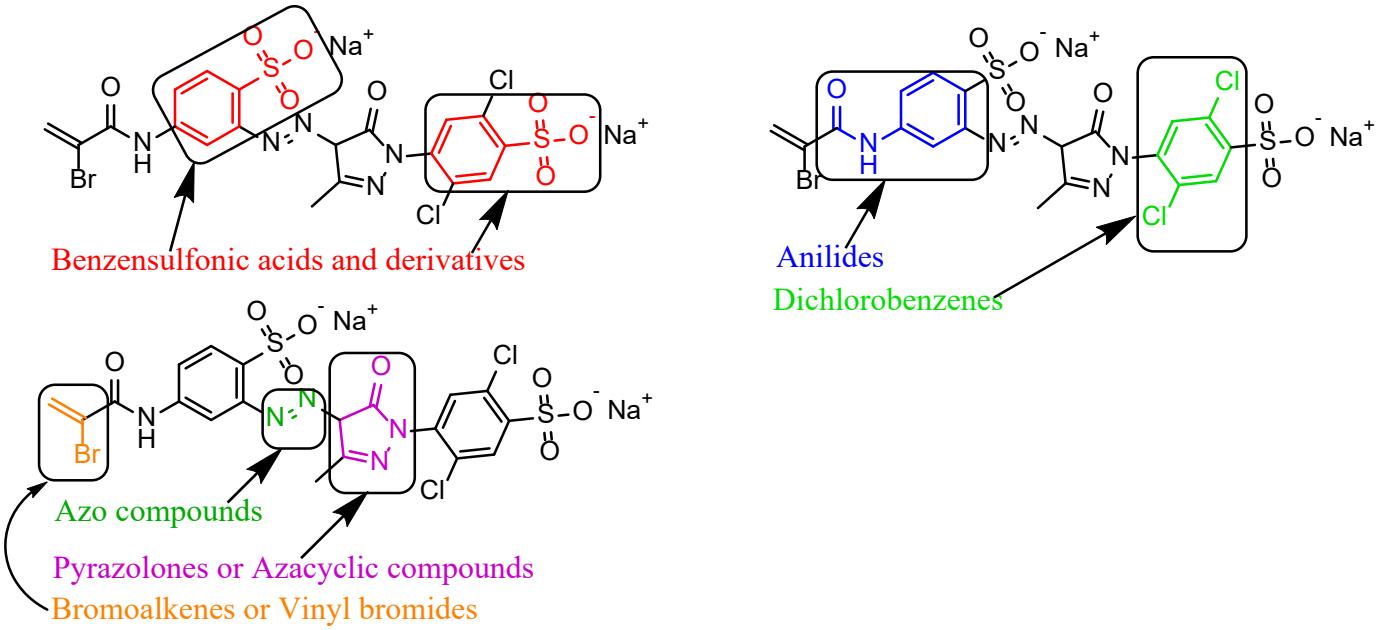


Figure A. Potential chemical classes of Lanasol Yellow 4G

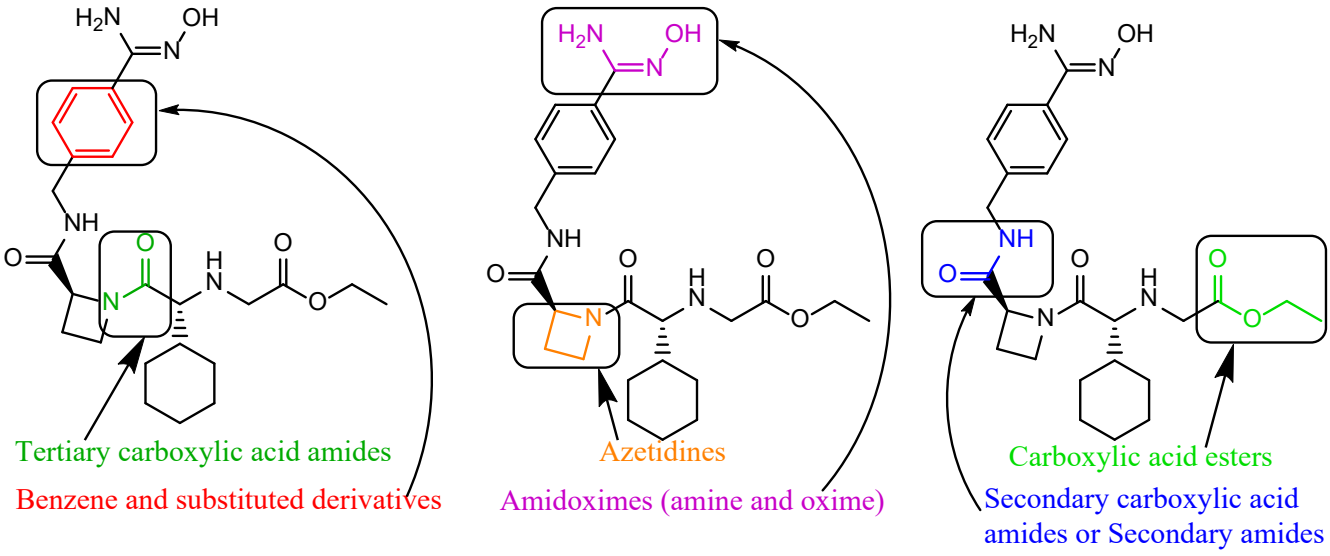


Figure B. Potential chemical classes of Exanta

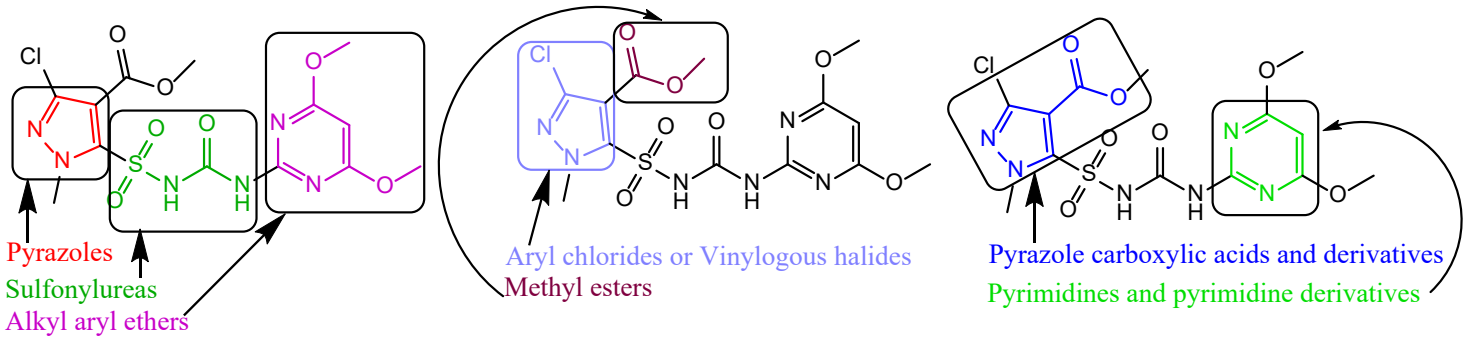


Figure C. Potential chemical classes of Halosulfuron-methyl

FDA notes that most compounds in the EDT DB are structurally complex, often containing multiple functional groups and/or intricate skeletons. As a result, assigning these compounds to a single chemical group is frequently not possible. To enable analysis of the chemical space covered by the EDT DB, we used the Classyfire software tool (http://classyfire.wishartlab.com/) to assign each compound to its respective chemical classes (Feunang et al., 2016). Given the structural complexity of many compounds, most can be categorized into multiple chemical classes. To address this, alternative chemical classifications are also provided in the EDT DB. For example, in the case of Lanasol Yellow 4G (see Figure A above), the EDT DB lists its chemical class as:

"Amino acids, peptides and analogues/Amino acids and derivatives/Alpha amino acids and derivatives **OR** Benzenesulfonic acids and derivatives **OR** 1-Sulfo, 2-unsubstituted aromatic compounds **OR** Anilides **OR** Benzenesulfonyl compounds **OR** Dichlorobenzenes **OR** N-arylamides **OR** Aryl chlorides **OR** Pyrazolones **OR** Sulfonyls **OR** Organosulfonic acids **OR** Azo compounds **OR** Secondary carboxylic acid amides **OR** Bromoalkenes **OR** Vinyl bromides **OR** Azacyclic compounds **OR** Organobromides **OR** Organochlorides **OR** Carbonyl compounds**."**

Here, **"OR"** denotes alternative chemical classifications, reflecting the compound's ability to fit into multiple categories based on its structure. FDA notes that while Classyfire places this compound into "Alpha amino acids and derivatives" likely because it contains functional groups or moieties that overlap with descriptors used for amino acids, Lanasol Yellow 4G does not contain amino acid(s) in its structure.

Below (see Figure D), to further demonstrate the difficulty of defining the EDT classes based on chemical classes, selected compounds from the EDT DB belonging to the chemical class of chlorobenzenes (compounds where one or more chlorine atoms are directly bonded to the carbon atoms of a benzene ring) and chlorobenzene derivatives will be utilized. 'Chlorobenzenes and chlorobenzene derivatives' are a subgroup within the chemical class of 'aryl chlorides' (compounds in which one or more chlorine atoms are directly bonded to an aromatic ring). In turn, 'aryl chlorides' is a subgroup within 'organochlorides', which, in turn, is a subgroup of 'organohalides'.

The compounds within the chemical group of chlorobenzenes and chlorobenzene derivatives display broad structural variation, hence broad range of toxicity, endpoints of toxicity, and mode of toxic action. Therefore, unsurprisingly, chlorobenzenes and chlorobenzene derivatives may be placed into any of the six EDT classes (see Figure D below after the reference), and they exhibit broad structural variation (with multiple possible additional chemical classifications/grouping) even within the same EDT class.

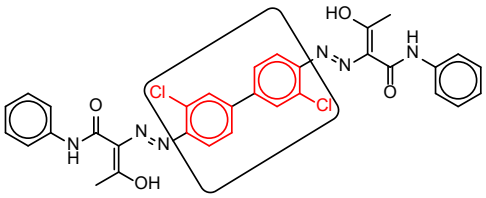
In summary, placing structurally complex compounds into a single chemical group and describing EDT classes solely in terms of the chemical classes they encompass are fraught with difficulties and, therefore, will not be attempted at this time. As mentioned earlier, the possible chemical classes of all compounds are provided in the EDT DB and allow for the search of specific chemical groups by interested parties for their own analysis.

The above information was added to section 4.6.1 (“Description of the Combined EDT DB”) of the EDT Peer Review document.

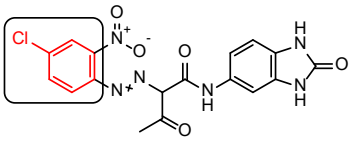
Reference:

Djoumbou Feunang Y, Eisner R, Knox C, Chepelev L, Hastings J, Owen G, Fahy E, Steinbeck C, Subramanian S, Bolton E, Greiner R, and Wishart DS. ClassyFire: Automated Chemical Classification With A Comprehensive, Computable Taxonomy. *Journal of Cheminformatics*, 2016, 8:61.
DOI: 10.1186/s13321-016-0174-y

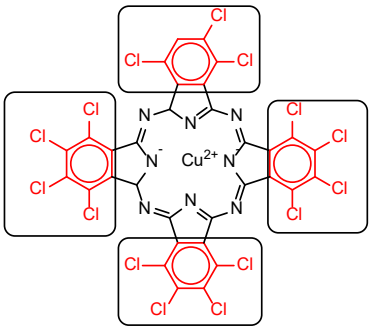
Examples of EDT Class I Chlorobenzenes and chlorobenzene derivatives:



Diarylanilide Yellow (CAS 6358-85-6)

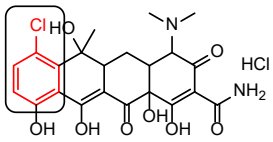


Copper phthalocyanine (CAS 147-14-8)

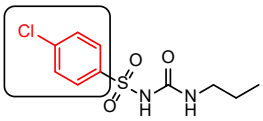


C.I. Pigment Orange 36 (CAS 12236-62-3)

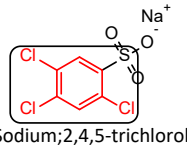
Examples of EDT Class II Chlorobenzenes and chlorobenzene derivatives:



Chlortetracycline hydrochloride (CAS 64-72-2)

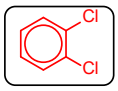


Chlorpropamide (CAS 94-20-2)

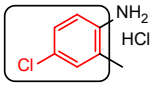


Sodium;2,4,5-trichlorobenzenesulfonate (CAS 53423-65-7)

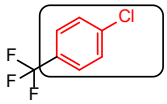
Examples of EDT Class III Chlorobenzenes and chlorobenzene derivatives:



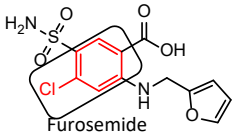
1,2-Dichlorobenzene (CAS 95-50-1)



4-Chloro-2-methylaniline HCl (CAS 3165-93-3)

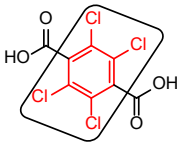


4-Chlorobenzotrifluoride (CAS 98-56-6)

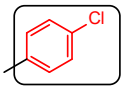


Furosemide (CAS 54-31-9)

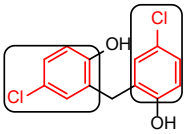
Examples of EDT Class IV Chlorobenzenes and chlorobenzene derivatives:



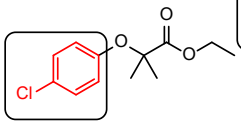
Chlorthal (CAS 2136-79-0)



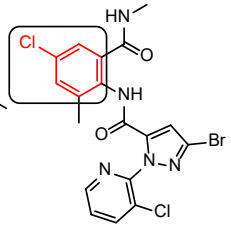
4-Chlorotoluene (CAS 106-43-4)



Dichlorophen (CAS 97-23-4)

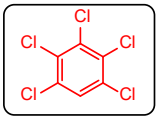


Clofibrate (CAS 637-07-0)

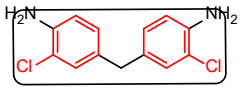


Chlorantraniliprole (CAS 500008-45-7)

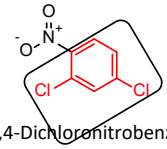
Examples of EDT Class V Chlorobenzenes and chlorobenzene derivatives:



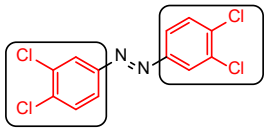
Pentachlorobenzene (CAS 608-93-5)



4,4'-Methylenebis(2-chloroaniline) (CAS 101-14-4)

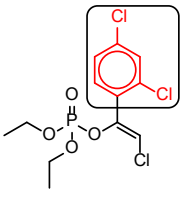


2,4-Dichloronitrobenzene (CAS 611-06-3)

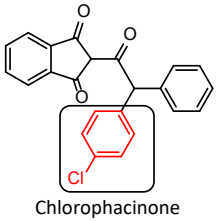


3,4,3',4'-Tetrachloroazobenzene (CAS 14047-09-7)

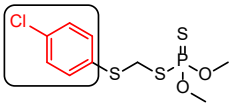
Examples of EDT Class VI Chlorobenzenes and chlorobenzene derivatives:



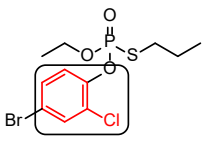
Chlorfenvinphos (CAS 470-90-6)



Chlorophacinone (CAS 3691-35-8)



Carbophenothion (CAS 786-19-6)



Profenofos (CAS 41198-08-7)

Figure D. Examples of chlorobenzenes and chlorobenzene derivatives from the EDT DB spanning the six EDT classes displaying broad structural variation

A peer reviewer raised a concern that TCDD is classified as Class V by the EDT, noting that the no-effect level (NEL) of TCDD is lower than the Class V TTC level, and therefore, suggesting that the Class V classification might not be "sufficiently protective." However, the FDA clarifies that, contrary to the peer reviewer's assertion, TCDD is classified under Class VI, not Class V, by the EDT. Class VI represents the highest toxic potential, aimed at capturing compounds with the most extreme toxic potentials. The EDT Class VI TTC level is 0.00053 µg/kg bw/day. According to the carcinogenicity study by Kociba et al. (1978), TCDD has a NEL of 0.001 µg/kg bw/day. Since this NEL is not lower than the Class VI TTC level, the TTC for this class is considered protective for TCDD. Therefore, the FDA maintains that TCDD is appropriately classified by the EDT.

Regarding the "overclassification" of endogenous phosphorylated compounds such as phytic acid: In a carcinogenicity study, only a lowest effect level (LEL) of 275 mg/kg bw/day was established for phytic acid, with no NEL reported (Hiasa et al., 1992). One primary concern with phytic acid in the diet is its capacity to bind minerals such as calcium, iron, and zinc, which may reduce their bioavailability (Hurrell and Egli, 2010; Kumar et al., 2010). This effect can be particularly significant if the dietary intake of these minerals is marginal, especially in populations with predominantly plant-based diets. However, most concerns about phytic acid toxicity stem from studies involving high doses or specific experimental conditions, rather than typical dietary exposure. While the FDA acknowledges that the EDT classification of phytic acid as Class III might be considered overprotective, based on NELs for some structurally closely similar compounds (see the EDT database, i.e., substances captured at the same sub-question), Class III was determined to be the most appropriate classification. Although the goal is to classify all compounds according to their true toxic potentials, some may have been placed in a higher class of concern due to their structural similarity to compounds that warrant such a classification. The FDA anticipates making further refinements to the tool as additional data becomes available in the future.

One of the peer reviewers observed that "skin sensitization and systemic hypersensitivity" appear not to be addressed by the EDT. The FDA clarifies that the EDT was developed solely to predict chronic toxic potential via the *oral* route of exposure. To ensure clarity on this point, the FDA has added the following statement to section 1.5 ("Applicability Domain and Definitions for Using the EDT"): "Additionally, please note that the EDT is designed specifically to predict the toxic potential of compounds through oral exposure."

One reviewer raised a concern about the decision tree approach, suggesting it might not always be the most suitable method. They proposed evaluating all alerts and applying the most conservative classification to enhance protection and transparency. Another reviewer questioned what happens if a molecule contains two "alerts," where the first alert is less toxic and leads to an early classification in the decision tree. They noted, "I fully understand the strength of the decision tree approach, but given the advancements we have, why not assess the molecule against all alerts and use the most conservative classification? This would ensure the classification is most protective and transparent."

The FDA acknowledges that compounds can possess multiple structural features

contributing to their toxicity, and it is crucial to classify compounds based on their most toxic structural features. To address this, we have implemented a “cross-checking” process in certain post-validation EDT questions. Specifically, when a user identifies the presence of specific structural features, we require them to cross-check these features against those described in other questions before finalizing the classification. These cross-checks are identified by the blue lines in Figure 2 of the EDT (section 4.5.4). The compound will be classified at the question with the highest EDT Class ensuring that the compound is classified according to its structural feature(s) with the highest toxic potential.

Regarding a peer reviewer’s comment of “It would be helpful to define whether (assuming they do) the six classes replace the requirement to deal with DNA reactive compounds and the Cohort of Concern separately.”: “Requirement to deal with DNA reactive compounds” separately will be a policy decision to be determined by individual agencies. The EDT is a scientific tool designed to inform safety and risk assessment by providing a prediction for oral chronic potency and establish conservative threshold exposures. It is neither intended to replace assessment of potential genotoxicity or carcinogenic risk assessment when such an evaluation is warranted nor represent an approach to satisfy a regulatory requirement stipulated in existing rules or regulations.

Agencies or programs may choose to limit the EDT’s applicability based on their own rules, laws, and program requirements.

References:

Kociba, R. J., Keyes, D. G., Beyer, J. E., Carreon, R. M., Wade, C. E., Dittenber, D. A., ... & Humiston, C. G. (1978). Results of a two-year chronic toxicity and oncogenicity study of 2,3,7,8-tetrachlorodibenzo-p-dioxin in rats. *Toxicology and applied pharmacology*, 46(2), 279-303.

Hiasa, Y., Kitahori, Y., Morimoto, J., Konishi, N., Nakaoka, S., & Nishioka, H. (1992). Carcinogenicity study in rats of phytic acid ‘Daiichi’, a natural food additive. *Food and chemical toxicology*, 30(2), 117-125.

Hurrell, R., & Egli, I. (2010). Iron bioavailability and dietary reference values. The American journal of clinical nutrition, 91(5), 1461S-1467S.

Kumar, V., Sinha, A. K., Makkar, H. P., & Becker, K. (2010). Dietary roles of phytate and phytase in human nutrition: A review. *Food chemistry*, 120(4), 945-959.

Question 4: Commonly, structurally related compounds (e.g., γ-diketones) can have common toxicological endpoint (in this case γ-diketone type neurotoxicity). Compounds that can either hydrolyze and/or metabolize to these compounds can exhibit the same type of toxicity. FDA aimed to capture hydrolytic and metabolic precursors of structurally related compounds with similar toxicities at numerous questions. Are there any questions where you can suggest any possible metabolic and/or hydrolytic precursors to the types of compounds addressed questions that are currently not mentioned/captured in the question?

Summary of general impressions:

The peer reviewers provided the following feedback regarding the EDT's coverage of hydrolytic and metabolic precursors:

1. The FDA was commended for the thoroughness of the EDT in capturing hydrolytic and metabolic precursors. While no specific omissions were identified, it was noted that this area was not a particular expertise for some reviewers.
2. There was a suggestion to consider proximate carcinogens, potentially including them in questions related to PAHs, to enhance the EDT's coverage.
3. The EDT was recognized for its effectiveness in classifying reactive intermediates accurately, with examples provided illustrating how the EDT assigns classes to compounds differently from other tools. Concerns were raised about whether hexane and its metabolites need to be assigned to EDT Class IV, given that long-term high concentration exposure is required to elicit toxicity, and whether a Cramer Class I classification might still be protective. Additionally, there was discussion about the practical issue of exposure to precursors rather than reactive intermediates, and the suggestion was made to explore linking EDT questions to third-party metabolism predictors to improve the tool's inclusiveness for reactive chemicals formed through metabolism.

FDA response:

The FDA thanks the peer reviewers for their thoughtful and detailed feedback on the EDT's coverage of hydrolytic and metabolic precursors. We greatly appreciate the recognition of the EDT's thoroughness and the valuable insights provided.

The FDA notes that i) at most questions, we aim to capture either structurally related compounds and/or compounds with the same mode of toxic action and endpoint of toxicity, and ii) the EDT was designed specifically to predict chronic *oral* toxic potential. For instance, the neurotoxic compound 2,5-hexanedione (CAS 110-13-4) did not yield a no-effect level (NEL) in a 90-day gavage study in rats, only a lowest effect level (LEL) of 0.54 mg/kg bw/day (Krasavage et al., 1980). The FDA derives a chronic LEL of 0.18 mg/kg bw/day by applying a duration adjustment factor of 3 to the subchronic LEL. This indicates that the chronic NEL is lower than this value, though the exact level remains undetermined. Similarly, for one of 2,5-hexanedione's metabolic precursors, 2-hexanone (CAS 591-78-6), a 392-day drinking water study in rats also failed to establish a NEL, showing only a LEL while clear signs of neurotoxicity were observed (O'Donoghue et al., 1978). Unfortunately, these oral studies used relatively high doses, making it challenging to determine presumptively safe chronic oral exposure levels. Additionally, for most other metabolic precursors, there are limited or no oral studies available. Consequently, to err on the side of caution, the FDA has classified 2,5-hexanedione and its metabolic precursors into Class IV. As more information on these and related substances becomes available, their classification may be updated accordingly.

Regarding PAHs, the FDA has included all known metabolic precursors and metabolites of

carcinogenic PAHs in the EDT. If readers are aware of additional metabolic precursors or metabolites of PAHs that should be considered for inclusion in question Q33, please email FDA at EDT@fda.hhs.gov with suggestions. The FDA welcomes all input that could help further refine the EDT questions.

References:

Krasavage, W. J., O'Donoghue, J. L., DiVincenzo, G. D., & Terhaar, C. J. (1980). The relative neurotoxicity of methyl-n-butyl ketone, n-hexane and their metabolites. Toxicology and applied pharmacology, 52(3), 433-441.

O'Donoghue, JL; Krasavage, WJ; Terhaar, CJ. (1978) A comparative chronic toxicity study of methyl n-propyl ketone, methyl n-butyl ketone, and hexane by ingestion. Eastman Kodak Company, Rochester, NY; Report No. 104657Y. Submitted under TSCA Section 8ECP; EPA Document No. 88-920008233; NTIS No. OTS0555051. Available from EPA Toxicological Review of 2-Hexanone (2009) at https://cfpub.epa.gov/ncea/iris/iris_documents/documents/toxreviews/1019tr.pdf

Question 5: Are the example structures provided after each EDT question correct and adequate for understanding what type of compounds the question aims to capture? Are there different or additional example structures for any of the questions that would help increase the understandability of the question?

Summary of general impressions:

The peer reviewers provided the following general impressions regarding the example structures in the EDT:

1. The inclusion of example structures is generally appreciated and deemed helpful for understanding the types of compounds targeted by each question. The structures are considered correct and adequate in most cases.
2. Some reviewers noted that the example structures can be challenging for non-chemists to interpret, suggesting that labeling carbon positions in compounds with substitutions would improve clarity.
3. The reviewers recommended additional enhancements, such as incorporating chemical or compound classes to define domains more clearly and linking these classes to modes of action or existing classification inventories.
4. Specific suggestions included using an example with an ellagic acid backbone for a particular question and considering the use of Kekulé structures for aromatic rings to maintain consistency.

Overall, the feedback highlights the value of the example structures while also pointing out areas for potential improvement to increase their usefulness and clarity.

FDA response:

FDA thanks the peer reviewers for their insightful and constructive feedback regarding the

example structures in the EDT. We greatly appreciate their recognition of the value these structures add in understanding the types of compounds addressed by each question. The peer reviewers' suggestions on enhancing the clarity of the examples are invaluable and we also appreciate the specific recommendations for improvements. Their input will help us refine the EDT to better serve its users and enhance its effectiveness.

Since the third point raised was already addressed elsewhere (see FDA's responses at question 3), the FDA will not revisit it in this section.

When the peer reviewers' input was initially provided for the pre-validation EDT, the peer reviewers did not have access to the post-validation EDT. In the post-validation EDT, we have increased the number of example structures provided after most questions to ensure that all questions, sub-questions, and sub-sub-questions include at least one example structure to assist in interpreting all chemical structure-based questions. Additionally, we have enhanced the number of skeleton structures provided (e.g., see the EDT Q1k) on bile acids) and, in certain examples, we highlighted specific structural features within the example structures to improve their utility.

Regarding ellagic acid, its backbone is included in Q12a). Next to this backbone, we have noted that "regardless of the substitution pattern on the ellagic acid backbone, if lactones are present as part of the ellagic acid backbone, respond no at Q12a. These compounds are evaluated at Q12e." In response to the peer review input, an example structure featuring the ellagic acid backbone has been added to Q12e), and a reference to this example has been included in Q12a).

Concerning the examples in Q47g), the structures in the post-validation EDT have been updated to incorporate the peer reviewer's suggestion. In the post-validation EDT, additional backbones have been included to help users identify related compounds for this question.

Question 6: Are there any congeneric groups that the EDT does not adequately address, but for which enough safety data exist that could serve as the basis of additional EDT questions to address these groups? If yes, please identify and provide all toxicological data for the congeneric group(s) that may form the basis of one or more additional questions. If possible, please propose the wording for such additional EDT questions. (Substances within a congeneric group are structurally and metabolically similar.)

Summary of general impressions:

The peer reviewers provided the following general impressions regarding congeneric groups and potential expansions for the EDT:

1. **Siloxanes**: There is notable interest in including siloxanes, which are frequently detected as extractables or leachables from food packaging materials. The availability of extensive toxicological data for both linear and cyclic siloxanes suggests that this congeneric group could benefit from additional EDT questions to better address their

safety.

2. **Metals and Organometals**: There is a recommendation to consider adding questions related to the solubility and pKa values of metals and organometals under physiological conditions. This would help refine the EDT's ability to assess these substances more accurately, particularly in understanding the variations in toxicity among different metal salts.
3. **Potential Areas for Expansion**: Several areas were suggested for potential expansion of the EDT, including:
   a) **Sugars and Amino Acids**: Especially those considered Generally Recognized As Safe (GRAS).
   b) **Polymer Components**: To encompass various polymer structures.
   c) **Botanicals**: To include a broader range of botanical classes.
   d) **Pharmaceuticals**: Leveraging extensive data and knowledge on mechanisms.
   e) **Nanomaterials**: Given their growing relevance and unique properties.
   f) **Biocides**: Including pesticide data and mechanisms.
   g) **Natural Toxins**: Such as mycocystins, which may be particularly relevant to food safety.
4. **Other Congeneric Groups**: There is no immediate identification of other congeneric groups with sufficient safety data that are currently not addressed by the EDT. However, it was noted that a thorough mapping exercise of existing data to the EDT questions could help clarify any gaps and refine the applicability domain of the tool.
5. **Mechanistic Approaches**: There is an acknowledgment that extending the EDT based solely on animal data could be restrictive. Therefore, integrating mechanistic approaches, such as using omics data or New Approach Methodologies (NAMs), could be valuable in extending the tool's applicability.

Overall, the feedback highlights both specific congeneric groups and general areas where the EDT could be enhanced, emphasizing the need for careful consideration of existing data and potential expansions to improve the tool's effectiveness.

FDA response:

We extend our sincere gratitude to the peer reviewers for their thoughtful and detailed feedback. Their insights into potential areas for expanding the EDT, including the inclusion of siloxanes, metals, and organometals, as well as other congeneric groups, are greatly appreciated.

Siloxanes are a subgroup of organosilicone compounds. FDA notes that while organosilicone compounds, including siloxanes, may, at first glance, appear to be addressed solely in Q5d(i)) and Q5d(ii)), which specifically inquire about the presence of silicon (Si), these compounds can also be classified under various other questions. In fact, the 28 organosilicones listed in the combined EDT database are categorized into Classes II, III, and IV across Questions 3, 5, 14, 19, 20, and 47. FDA recognizes that the presence of Si alone is not always a decisive factor in determining the toxic potential of organosilicone compounds. Consequently, although it might not be immediately evident, the post-validation EDT accurately classifies organosilicones based on their most toxic

structural features. However, FDA remains open to refining these classifications as new oral toxicity data becomes available in the future.

One peer reviewer suggested that FDA consider adding questions about the solubility and pKa values of metals and organometals under physiological conditions. This, they argued, would enhance the EDT's ability to more accurately assess these substances, especially in distinguishing variations in toxicity among different metal salts. FDA notes that while the EDT does address some metals as counterions to organic compounds and organometals, its primary focus is on organic compounds, with the goal of classifying them based on their structural features. Currently, there is limited publicly available data on subchronic and chronic oral toxicity for organometals. Therefore, FDA is not able to expand the questions addressing these types of compounds in the current paper version of the EDT at this time. However, we may consider including this suggestion (i.e., employing questions on solubility and pKa) in future updates after more consideration. This update may be more appropriate for the software version of the tool.

FDA acknowledges that relying solely on animal data to extend the EDT could be restrictive. Therefore, integrating mechanistic approaches, such as omics data or New Approach Methodologies (NAMs), as suggested by one of the reviewers, could enhance the tool's applicability. While the current EDT questions considered these types of data to some extent during their development, FDA plans to incorporate more of these approaches in future updates. This aligns with FDA's goal of reducing reliance on animal testing.

FDA notes that the EDT already includes questions that address sugars, amino acids, and their related substances, which are expected to be presumptively safe (see EDT Q1). In the post-validation EDT, we have expanded the scope to include a broader range of natural toxins compared to the pre-validation version (see Q6). For example, in the post validation EDT, we added questions designed to capture aflatoxins (Q6d(i)), brevetoxins (Q6d(ii)), ciguatoxins (Q6d(ii), and ochratoxins (Q6d(vi)). Another example of an added question to address natural toxins is Q6b(iv) that captures compounds with azetidine rings such as azetidine-2-carboxylic acid, which is found in certain plants and can act as a natural toxin by interfering with protein synthesis. For polymers, the EDT currently assesses the safety of a limited range of hydrolyzable polymers as well as the specific monomeric units of various polymers. FDA aims to enhance the EDT's capability to evaluate the toxic potential of polymers more comprehensively in future updates.

Biocides are chemical or biological substances intended to control, prevent, or eliminate harmful organisms such as bacteria, fungi, algae, viruses, or pests. The pre-validation EDT covered a broad range of biocides, but the post-validation EDT introduces additional questions to better address these compounds. For instance, post-validation EDT Q6e(iii)) is designed to capture various neonicotinoids, which are synthetic insecticides modeled after nicotine. Neonicotinoids are extensively used in agriculture to manage a wide range of pests, including those affecting crops, garden plants, and ornamental plants.

With respect to the comment regarding nanomaterials, the oral toxicity of a compound can

vary with its particle size because of differences in surface area-to-volume ratio, absorption efficiency, dissolution rate, and distribution in the body. Smaller particles may exhibit increased toxicity due to higher absorption and systemic exposure, while larger particles might be less toxic due to reduced absorption. Understanding these effects is essential for assessing the safety and potential risks of materials with different particle sizes. Currently, our knowledge is limited by a lack of data on how these factors affect toxicity. As a result, the EDT is not equipped to predict changes in toxic potential based on particle size and was not designed to classify nanomaterials. We may consider introduction of this concept in futures version of the EDT.

While the pre-validation EDT questions addressed some pharmaceuticals and the post-validation EDT expanded to include more, the EDT was not designed to comprehensively sort all pharmaceuticals based on their chronic oral toxic potential. Several factors contribute to this limitation:

1. **Structural Complexity**: Pharmaceuticals exhibit a vast range of structural variations and complexities, making it challenging, if not impossible, to accurately predict their chronic toxic potential. That stated, numerous computational toxicology models are currently available for predicting the toxic potentials of pharmaceuticals under investigation. Our aim was not to create another one of these tools.
2. **Existing Safety Testing Requirements**: Pharmaceuticals must undergo extensive preclinical and clinical safety testing before approval. The EDT was not intended to replace these rigorous safety assessments of active pharmaceutical ingredients but rather to complement them. That stated, a large number of excipients and potential impurities are included in the EDT DB and are addressed by the EDT questions.
3. **Data Availability**: The EDT was developed using only publicly available safety data. Preclinical safety data for pharmaceuticals are often proprietary and not publicly accessible. When available, these data are frequently too limited to determine study duration, no-effect levels, or appropriate EDT classification.

   FDA anticipates that with more detailed preclinical safety data becoming available in the future, the EDT may be updated to address a broader range of pharmaceuticals.

Question 7: Should any questions be further subdivided to ensure a more refined grouping of related substances? If yes, please suggest wording for the refined question(s) and provide the data justifying the suggestion.

Summary of general impressions:

The general impression from the peer reviewers is that the current grouping of substances in the EDT is well-defined and exhibits a high level of expertise and thoughtfulness. Several reviewers expressed satisfaction with the existing structure and did not suggest obvious refinements at this time. However, one reviewer noted that the adequacy of the groupings could be further assessed after examining additional data. Additionally, there was a suggestion to refine the grouping for metals and organometals by incorporating questions about their solubility and pKa values, which could help address the variability in toxicity among different metal salts.

FDA response:

FDA thanks the peer reviewers for their feedback on whether any EDT questions should be further subdivided to ensure a more refined grouping.

Regarding incorporating pKa and solubility into the EDT questions, please see FDA's response to question 6.

FDA acknowledges that while peer reviewers did not suggest the need for extensive refinements of the pre-validation EDT, the results of the external validation of the pre-validation EDT indicated that significant refinements were necessary. These refinements, along with the scientific basis and reasoning, were provided to the peer reviewers in Phase II, in section 4.4 ("The External, Independent Validation of the EDT") of the peer review document.

Question 8: Are there any terms used in the EDT questions that should be added to the guidelines and definitions section to help users of the EDT? If yes, what additional terms should we define?

Summary of general impressions:

The peer reviewers provided the following general impressions regarding the guidelines and definitions section of the EDT:

1. **Additional Definitions**: There was a recommendation to define specific terms such as "conjugated," "dimer," "organyl," and "connector" to enhance the understanding of the questions.
2. **Isomeric Forms**: The need to include definitions for common isomeric forms, such as stereoisomers and enantiomers, was highlighted as it would aid in understanding questions involving compounds like flavonoids.
3. **Applicability Domain**: One reviewer noted that while the chemistry definitions are well-covered, it may be beneficial to include an explanation of the EDT's applicability domain in the guidelines and definitions section.

One of the peer reviewers also suggested in their response that the detailed explanations currently found in Appendix 1 should be integrated directly into the EDT questions for better clarity and accessibility.

Overall, the feedback emphasizes the need for only a few additional definitions for terms and concepts relevant to the questions.

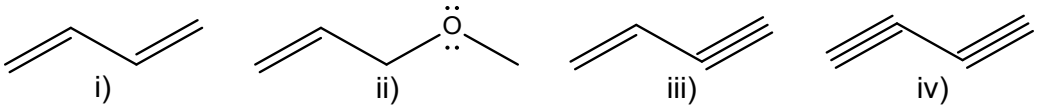
FDA response:

We would like to extend our sincere thanks to the peer reviewers for their valuable feedback regarding the addition of terms to the definition section of the EDT. Their

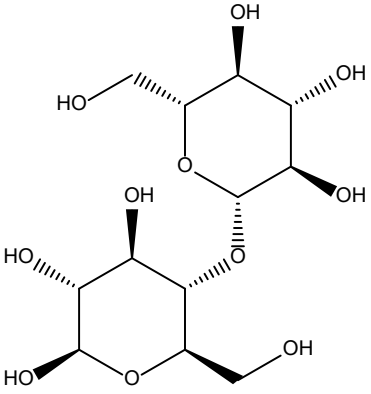
suggestions for including definitions of key terms will greatly enhance the clarity and usability of the tool.

In response to the suggestions, FDA added the following definitions to sections 1.5 and 4.5.2 (sections providing the definitions for the pre- and post-validation EDT, respectively):

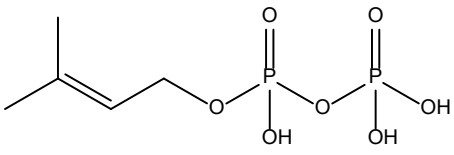
In chemistry, **conjugated** refers to a specific arrangement of alternating single and double bonds within a molecule. This arrangement involves the overlap of p-orbitals across adjacent bonds, allowing for the delocalization of electrons across the entire system. When a double bond is adjacent to a single bond and the single bond is connected to a nitrogen or oxygen atom with lone pair electrons, these lone pairs can participate in conjugation. The lone pair on the nitrogen or oxygen can overlap with the π-system of the adjacent double bond. This interaction is often referred to as lone pair conjugation or n → π interaction and can affect the molecule's electronic structure, influencing properties such as reactivity and stability. In an alternating double bond-single bond-triple bond configuration, true conjugation does not occur because the triple bond does not participate in p-orbital overlap with the double bond in the same manner. However, there can be some electronic interaction between the double and triple bonds, though it is generally not as extensive or stabilizing as true conjugation and may affect the molecule's properties. Similarly, in a triple bond-single bond-triple bond configuration, the triple bonds do not conjugate with each other through the single bond. While there is no true conjugation here either, there may be some electronic effects or inductive interactions that can influence the molecule's stabilization and chemical properties. For the purposes of the EDT, to simplify its language, the following configurations are referred to as conjugation: i) double bond-single bond-double bond, ii) double bond-single bond-nitrogen or oxygen atom with lone pair of electrons, iii) double bond-single bond-triple bond, and iv) triple bond-single bond-triple bond.



A **dimer** refers to a molecule that is formed by the combination of two identical or similar smaller molecules (monomers) through a chemical reaction. Examples:



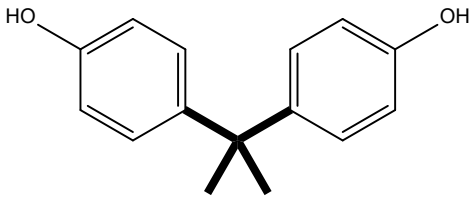
cellobiose (dimer of glucose)



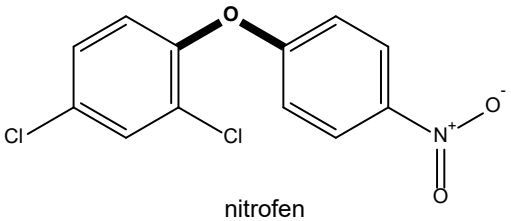
dimethylallyl diphosphate (a phosphate dimer)

Organyl refers to a general class of organic fragments that contain a carbon-based structure. Specifically, it often denotes an organic group or substituent derived from an organic molecule. While organyl can apply to various types of organic groups or substituents, such as alkyl groups (e.g., methyl, ethyl), aryl groups (e.g., phenyl, tolyl), or more complex structures, the organyl group or substituent is always based on organic carbon structures.

A **connector** is a structural element that links two distinct rings or fragments in a molecule through chains and/or functional groups, without fusing the rings together. Examples:



bisphenol A
(connector is **bolded**)



nitrofen
(connector is **bolded**)

As stated in FDA's response to question 1, FDA added a paragraph defining the applicability domain of the EDT and clarified that the tool is designed to predict the toxic potential of compounds through oral exposure in section 1.5 ("Applicability Domain and Definitions for Using the EDT.")

While one of the peer reviewers suggested to define the terms stereoisomer and enantiomer, these terms were not defined as they were never used in any of the EDT questions.

Question 9: Has FDA clearly explained where the toxicological data found in the EDT DB were collected from? If not, what additional information should we provide?

Summary of general impressions:

The peer reviewers generally praised the FDA for the effort and transparency in data collection for the EDT. However, they identified some areas for improvement. They recommended including a fully documented and curated database with clear references to the sources of data, similar to examples like the COSMOS DB with the EDT peer review document. In their opinion, this would enhance transparency and future-proofing of the data.

Suggestions included:

1. Clearly explaining the sources and criteria for toxicological data collection, including the search terms and databases used (e.g., PubMed, Google).

2. Providing additional details on how information was gathered, particularly for food contact substances and the inclusion of study details.
3. Incorporating a table or additional information about the number of chemicals from various sources and the curation process to ensure unique substances.
4. Addressing issues with CAS numbers and the completeness of chemical descriptions.
5. Provide fuller details on the criteria for inclusion and exclusion, especially for specific groups of chemicals such as organosilicon substances, endocrine disruptors, and nanomaterials.

These enhancements would provide a more comprehensive understanding of the data sources and improve the clarity of the EDT's development process.

FDA response:

FDA thanks the peer reviewers for their valuable feedback on data collection, transparency, and database documentation. Their insights are instrumental in refining our approach and improving the EDT.

FDA believes transparency is crucial for gaining public trust, ensuring data integrity, and facilitating informed decision-making. Therefore, FDA will release the combined EDT database (DB) along with the peer review document, the charge questions, and FDA's response to the peer reviewers' comments on FDA's publicly available peer review website for full transparency. The combined EDT DB was also provided to the peer reviewers. This DB contains the primary reference, whenever available, for all safety data used to support the EDT. FDA notes that in some cases, only secondary references are provided as no primary reference was available. Nonetheless, all sources of toxicological data are disclosed for every substance. Toxicological data for a large number of substances was available from various sources. In certain cases, the different sources may not have agreed on the NEL and LEL. These were discussed in the comment column for the safety data, and, in these cases, the various data sources were listed to enable the users to review these evaluations. As an example, the relatively simple case of Chlorpyrifos-methyl is presented below.

Chlorpyrifos-methyl (CAS 5598-13-0) is an organophosphate pesticide used to control insects in stored grain and other food products. It is a methyl ester derivative of chlorpyrifos, which is another widely known organophosphate insecticide. Chlorpyrifos-methyl has a similar mode of action to chlorpyrifos, targeting the nervous systems of pests by inhibiting the enzyme acetylcholinesterase, which is crucial for nerve function.

Chlorpyrifos-methyl was tested in chronic dietary toxicity/oncogenicity study in rats at 0, 0.05, 0.1, 1, or 50 mg/kg bw/day (Barna-Lloyd et al., 1991). The original study report by Barna-Lloyd et al. (1991) is not publicly available. A literature search for this substance

yielded that JMPR (JMPR, 2009), EPA (EPA, 2015), and EFSA (EFSA, 2019) evaluated the safety of this substance and considered this study in their evaluations.

According to JMPR, "A NOAEL[3] of 1 mg/kg bw per day can be determined for this study, based on decreased brain cholinesterase activity, increased adrenal weights and associated histopathology at 50 mg/kg bw per day. Animals were fasted prior to termination; it is therefore possible that terminal cholinesterase inhibition was underestimated in this study. However, reassurance is gained from cholinesterase results in a previous 2-year rat study (Barna-Lloyd, Szabo & Davis, 1991)." JMPR goes on stating that "A histopathology review panel performed a "blind" reading of the adrenal slides from the study of Barna-Lloyd, Szabo & Davis (1991). The review included a scoring for severity of vacuolation that was absent from the original study. The review panel concluded that the findings of adrenal vacuolation at 1 mg/kg bw per day and below were consistent with background findings and that the only dose producing clear effects was the top dose of 50 mg/kg bw per day (Table 19) (Bruner & Gopinath, 2000)."

According to EPA, "In the rat combined chronic toxicity/ carcinogenicity study (MRID 42269001), the NOAEL and LOAEL for RBC ChEI were established at 1.0 and 50.0 mg/kg/day, respectively, but there were no indications of clinical signs. At 50 mg/kg/day in the rat, body weight decreases, alterations in the adrenals (increased weight, slight to moderate vacuolation with lipid accumulation in the zona fasciculata) were observed."

And finally, according to EFSA, "The main effect following short- to long-term repeated oral administration of chlorpyrifos-methyl was the inhibition of acetylcholinesterase (AChE) activity, which, at high-dose levels, was leading to endogenous cholinergic overstimulation resulting in typical cholinergic symptoms. Erythrocyte (red blood cell (RBC)) AChE inhibition was the critical effect in all studies conducted with rats, mice and dogs. Additionally, the adrenals (increased weight, hypertrophy and vacuolation of cells of the zona fasciculata) were identified as target organ of chlorpyrifos-methyl in rats. The relevant no observed adverse effect level (NOAEL) for short-term toxicity was 0.65 mg/kg body weight (bw) per day from the 28-day toxicity study in mice and 0.1 mg/kg bw per day for long-term exposure from the 2-year study in rats[4] based on significant decrease of RBC AChE activity in both studies and adrenal toxicity upon long-term exposure in rats only."

FDA assigned a NOAEL of 1 mg/kg bw/day to the chronic dietary toxicity/oncogenicity study of chlorpyrifos-methyl based on the available evidence and a review of international evaluations. While EFSA identified effects on erythrocyte acetylcholinesterase (RBC AChE) activity and adrenal glands at lower doses (0.1 and 1 mg/kg bw/day), both JMPR and EPA determined that the effects on RBC AChE activity were inconsistent over time and lacked a clear dose-response relationship. Importantly, a histopathology review panel

[3] No-observed-adverse-effect-level
[4] Barna-Lloyd et al., 1991

concluded that adrenal vacuolation observed at these doses was consistent with background findings, reaffirming that the only clear adverse effects occurred at the top dose of 50 mg/kg bw/day. Therefore, FDA aligns with JMPR and EPA in considering the NOAEL for this study to be 1 mg/kg bw/day, as it reflects the highest dose without consistent adverse effects and provides a scientifically robust basis for regulatory decisions.

The above short example was added to section 4.3.5 (“Selection of the Best Representative Study for Each Substance in the External Validation Database”) of the EDT Peer Review document.

Reference:

Barna-Lloyd, T., Szabo, J.R. & Davis, N.L. (1991) Chlorpyrifos-methyl (Reldan insecticide): chronic dietary toxicity/oncogenicity study in rats. Unpublished report No. from Dow Chemical Co., TX, USA. Report No. TXT:K-0461-93-031. Submitted to WHO by Dow AgroSciences, Midland, MI, USA. Available from JMPR (2009) Chlorpyrifos-methyl Monograph at https://apps.who.int/pesticide-residues-jmpr-database/Document/101. Also available from EPA (2015) Chlorpyriphos-Methyl: Human Health Draft Risk Assessment (ORA) for Registration Review at https://www.regulations.gov/document/EPA-HQ-OPP-2010-0119-0020 and EFSA (2019) Updated statement on the available outcomes of the human health assessment in the context of the pesticides peer review of the active substance chlorpyrifos-methyl at https://www.efsa.europa.eu/en/efsajournal/pub/5908

While one peer reviewer noted that "Section 2.2 provides criteria for data collection. However, the source of the toxicological data is not explained until section 4.2 Creation of the External Validation Database," FDA clarifies that section 2.1 also lists the sources of toxicological data for the pre-validation (or “original”) EDT database.

Another peer reviewer inquired, “When discussing searching the literature, please mention what search terms were used and what was used to search (i.e., PubMed, Google). For example, what types of information/studies were searched for, just subchronic and chronic?” In response, as detailed in sections 2.1 and 4.2, FDA searched multiple publicly available databases, including, but not limited to, the US Environmental Protection Agency Integrated Risk Information System (EPA IRIS), US EPA High Production Volume Information System (EPA HPVIS), US EPA Pesticides: Reregistration, California EPA (CalEPA), and the European Chemicals Agency (ECHA). For these searches, we utilized CAS numbers, common names, and IUPAC names of chemicals. Additionally, we conducted searches using Google, Google Scholar, and PubMed. The search terms included phrases such as “safety of [chemical name],” “toxicity of [chemical name],” “mode of action of [chemical name],” “carcinogenicity of [chemical name],” with [chemical name]

representing both common and IUPAC names for all chemicals. The search terms were added to section 2.1 (“Creation of the Original EDT Chemistry, Toxicity, and Metabolism Database (EDT DB)”).

The same reviewer asked, “Where was information on food contact substances gathered from?” FDA conducted a comprehensive search for toxicological data on food contact substances across all listed data sources to ensure thoroughness. This approach was adopted because substances often have multiple applications, even though we did not anticipate finding these substances in certain databases, such as those maintained by the EPA.

Additionally, the same reviewer inquired, “When mentioning study details included, did the substance need to have all this information to be included in the EDT DB?” Yes, FDA required that all specified study details (including species, strain, sex, duration, dose levels, frequency of dosing, purity of the test article, and details on adverse and adaptive effects observed) be included in the EDT DB to ensure completeness and transparency.

Another peer reviewer suggested that providing information on the number of chemicals from different data sources would have been useful. As previously mentioned, for every substance FDA searched all potential data sources (e.g., ECHA DB, various EPA DBs, and Google Scholar). Often, safety data for the same substance were available from multiple sources, and conclusions such as NEL and LEL varied. All relevant data, opinions/conclusions, and references were discussed and listed in the EDT DB. Because multiple sources of data were captured for the same substance, FDA captured the data in such a way that users can analyze the data and determine counts of compounds from different sources.

The reviewer also requested more information on the "curation to get unique substances." To ensure uniqueness in the EDT DB, FDA first identified duplicates based on CAS numbers, names, and SMILES codes, deleting redundant entries. Once classified, compounds were grouped by question, sub-question, and sub-sub-question. As non-toxic or low-toxic potential counterions are disregarded by the EDT, various salt forms and the neutral form of the same compound are normally classified under the same question, sub-question, or sub-sub-question. For salts, the presence of other salt forms or the neutral form was manually examined within the same question, sub-question, and sub-sub-question. The form with the best representative study was chosen to represent the substance and its various salt and neutral forms. FDA expanded section 4.3.1 of the EDT peer review document to provide more details on the curation of unique substances.

The same peer reviewer also noted that “The issue of inaccurate and multiple CAS numbers, incomplete description of the chemical, etc. is described in the External Validation Database but not for the pre-validated EDT.” and “Missing is also the extensive

discussion found in the External Validation Database but not for the pre-validated EDT on the approach used when administration of the chemical was not every day or when the compound had a lower purity." FDA acknowledges that it was an oversight not to provide information on adjusting NELs for dosing schedule and purity for the pre-validation ("original") EDT DB; this information was initially included only for the validation DB. To address this oversight, FDA has added the relevant details to section 2.2 ("Criteria for Data Selection and Derivation of Duration, Purity, and Dosing Schedule Adjusted NELs.") To address the first point raised, certain compounds may have more than one CAS numbers.[5] To help minimize duplicate entries in the EDT DB for the same substance under different CAS numbers, in addition to checking for duplicate CAS numbers, we also scanned the DB for duplicate common and IUPAC names and SMILES codes. This information was added to section 2.1 ("Creation of the Original EDT Chemistry, Toxicology, and Metabolism Database (EDT DB)").

Lastly, the reviewer recommended fuller details on the criteria for inclusion and exclusion, especially for specific groups like organosilicon substances, endocrine disruptors, and nanomaterials. FDA clarifies that all compounds either excluded from or included in the EDT applicability domain are listed in sections 1.5, 4.3.2, and 4.5.2 of the EDT peer review document. The EDT excludes unhydrolyzable polymers, proteins, elements, inorganic substances, and substances with undefined structures. Sections 1.5 and 4.5.2 also note that the EDT is not designed to estimate safe intake levels (i.e., TTCs) based on particle size and is not suitable for classifying nanomaterials.

Question 10: Has FDA clearly explained the study selection criteria and provided adequate information and/or data to support its opinion that these criteria are appropriate for data inclusion in the DB? If not, what additional information should we provide?

Summary of general impressions:

The peer reviewers generally found that FDA provided a clear explanation of the study selection criteria for data inclusion in the EDT database, but they identified areas for improvement to enhance transparency and user understanding. They appreciated the detailed criteria but suggested that additional information and clearer presentation would benefit users.

Specific Requests and Notes:

[5] When a test item is a mixture (e.g., cis-trans or optical isomers), each component may be assigned a unique CAS number. CAS numbers can also vary for technical mixtures, commercial grades, or substances with specific impurities. In some instances, a single substance may have more than one CAS number, one typically classified as the "preferred" CAS number and one or more "alternate" CAS numbers. Consequently, some substances in the EDT database appear with more than one CAS number.

1. **Parenteral Routes**: Consider combining data from different parenteral routes (subcutaneous, intravenous, intraperitoneal) and analyzing them collectively for the parenteral route.
2. **Conflicting Data**: Address and discuss conflicting data interpretations and how such issues were managed, as seen for the External Validation Database.
3. **Selection Criteria**: Provide more detail on the criteria for including or excluding substances, particularly for groups like organosilicon substances, endocrine disruptors, and nanomaterials.
4. **Toxicological Endpoints**: Clarify definitions and guidelines for toxicological endpoints. Provide clear distinctions between NOAEL and NOEL[6], and specify how adversity and non-adverse effects are considered.
5. **Benchmark Dose Lower Confidence Limit[7] (BMDL) Values**: Explain the role of BMDL values in the study selection and the decision-making processes when both BMDL and NO(A)EL[8] values are available.
6. **Study Details**: Provide more details on study inclusions, such as:
   a) Search terms and databases used for literature search.
   b) Handling of substances without identified NELs and conversion of LELs.
   c) Adjustment of NELs for purity and dosing schedule.
   d) Species, duration, statistical significance, and adverse effects evaluation.
7. **Improvement Suggestions**:
   a) Add headings to section 2.2 to improve readability.
   b) Clarify the treatment of offspring vs. parental NELs.
   c) Discuss the rationale behind not distinguishing between genotoxic and non-genotoxic compounds in the criteria.
   d) Explain what is meant by “uncatalyzed metabolism”.
   e) In section 2.2, FDA mentions studies with “limited reporting”. Explain what “limited” refers to.
   f) In section 2.2, FDA talks about species differences. Edit for clarity.

These suggestions aim to enhance the clarity, completeness, and usability of the information provided in the EDT database.

FDA response:

FDA appreciates the peer reviewers' insightful feedback and suggestions for improving the clarity and completeness of the study selection criteria and database documentation. Their detailed comments will help enhance transparency and ensure the robustness of the EDT database.

[6] No-observed-effect-level
[7] The BMDL represents the lower bound of a confidence interval for the benchmark dose, which is the dose at which a predefined increase in effect (like a certain percentage increase in adverse effects) is observed.
[8] For the purposes of the EDT, NO(A)EL is considered the same as NEL (no effect level) and, as such, it encompasses both NOAEL and NOEL.

For clarity, ease of presentation, and due to length of FDA's responses, the specific suggestions and comments above will be restated below, with FDA's responses added in blue right after on a new line.

1. **Parenteral Routes**: Consider combining data from different parenteral routes (subcutaneous, intravenous, intraperitoneal) and analyzing them collectively for the parenteral route.
   Less than 4% of the studies in the combined EDT DB involved parenteral administration. Parenteral studies were included only when oral studies were unavailable or unsuitable (e.g., studies with only one or two animals per dose or substances that could not be administered orally due to their physicochemical properties). The EDT was specifically designed to assess *oral* toxic potential, so combining and analyzing only parenteral data would not enhance the tool's effectiveness for predicting oral toxicity. FDA, if resources allow, aims to either develop similar decision trees for dermal and inhalational routes but also encourages others to undertake this task.
2. **Conflicting Data**: Address and discuss conflicting data interpretations for the pre-validation (original) EDT DB and how such issues were managed, as seen for the External Validation Database.
   FDA notes that conflicting data interpretations for the pre-validation (original) EDT DB were addressed and discussed in section 2.1. Please refer to the paragraph beginning with "For many substances, authoritative bodies do not agree as to the NEL and LEL…" for detailed information.
3. **Selection Criteria**: Expand on the criteria for including or excluding substances, particularly for groups like organosilicon substances, endocrine disruptors, and nanomaterials.
   This issue was addressed at Q9. Briefly, all compounds either excluded from or included in the EDT applicability domain are listed in sections 1.5, 4.3.2, and 4.5.2 of the EDT peer review document. The EDT excludes unhydrolyzable polymers, proteins, elements, inorganic substances, and substances with undefined structures. Sections 1.5 and 4.5.2 also note that the EDT is not designed to estimate safe intake levels (i.e., TTCs) based on particle size and is not suitable for classifying nanomaterials. No other groups of compounds are excluded.
4. **Toxicological Endpoints**: Clarify definitions and guidelines for toxicological endpoints. Provide clear distinctions between NOAEL and NOEL, and specify how adversity and non-adverse effects are considered.
   Certain studies produced only NOEL or NOAEL values, while others included both. When only one of these values was available, it was used for calculating the class TTC. Since NOEL encompasses all types of effects (both adverse and non-adverse), and NOAEL specifically excludes adverse effects, when both NOEL and NOAEL were provided, the NOAEL was selected for TTC calculations. Providing a comprehensive and detailed overview of what constitutes an adverse effect and its relevance to

humans is beyond the scope of this project. To determine whether an effect is adverse, non-adverse, adaptive, or an artifact, FDA consulted various sources, including Pandiri et al. (2017) [Toxicologic Pathology, 45(1), 238-247]. Consequently, the peer review document includes only examples of adverse effects and a specific example of an adverse effect seen in laboratory animals but deemed not relevant to humans. FDA also notes that when reviewing all available studies for a specific substance, especially older evaluations, we incorporated current knowledge on whether the effect is adverse and the relevance of adverse effects observed in laboratory animals to humans when assigning a NOAEL or NOEL to a study.

5. **BMDL Values**: Explain the role of BMDL values in the study and the decision-making process when both BMDL and NO(A)EL values are available.
   For most studies, no BMDL values were available, and, in many cases, the data were not suitable for BMDL calculation. FDA reviewed all available oral studies for the 3,100+ substances in the combined database. Determining the suitability of each study for BMDL calculation and performing such calculation were beyond the scope of this project and beyond our available resources. Therefore, for consistency, we only used NO(A)EL values for TTC calculations even in the somewhat rare instances where BMDL values were available. Moreover, this approach is in line with how others (e.g., Munro et al.) derived TTCs.
6. **Study Details**: Provide more details on study inclusions, such as:
   a) Search terms and databases used for literature search.
      This point was also raised and addressed at question 9. Briefly, as detailed in sections 2.1 and 4.2, we searched multiple publicly available databases, including, but not limited to, the US Environmental Protection Agency Integrated Risk Information System (EPA IRIS), US EPA High Production Volume Information System (EPA HPVIS), US EPA Pesticides: Reregistration, California EPA (CalEPA), and the European Chemicals Agency (ECHA). For these searches, we utilized CAS numbers, common names, and IUPAC names of chemicals. Additionally, we conducted searches using Google, Google Scholar, and PubMed. The search terms included phrases such as “safety of [chemical name],” “toxicity of [chemical name],” “mode of action of [chemical name],” “carcinogenicity of [chemical name],” with [chemical name] representing both common and IUPAC names for all chemicals. The search terms were added to section 2.1 Creation of the Original EDT Chemistry, Toxicity, and Metabolism Database (EDT DB).
   b) Handling of substances without identified NELs and conversion of LELs.
      As mentioned in the first paragraph of section 3.3, "Derivation of the Pre-validation EDT TTC Levels," in the peer review document, the pre-validation (original) EDT DB, containing 1,628 NELs, was robust enough for its intended purpose, which is why we chose not to derive NELs from LELs. Additionally, FDA did not generate NELs from LELs for studies lacking NELs in the External Validation Database.
   c) Species, duration, statistical significance, and adverse effects evaluation.
      i) Regarding the species included in the combined EDT DB, studies were

conducted using the following: rat (2,574), dog (313), mouse (202), rabbit (22), monkey (19), hamster (6), pig (5), and unspecified (1), with the numbers in parentheses indicating the count of substances. This information was added to section 4.6.1 (“Description of the Combined EDT DB”) of the peer review document.

ii) For study duration, while we included studies with a duration of less than 84 days for Classes I-V when longer studies were unavailable, we did not use NELs from studies lasting less than 84 days for Class I-V TTC calculations. In Class VI, 71% of representative studies lasted at least 84 days, nearly 15% lasted between 28 and 83 days, almost 8% ranged from 10 to 27 days, and less than 7% were shorter than 10 days (with only two studies lasting 1 day). For studies with a duration of less than 84 days that produced NELs, a duration adjustment factor of 10 was applied to derive chronic NELs, representing the most conservative duration factor used. As detailed in the EDT peer review document, we utilized studies with durations of less than 84 days to calculate the Class VI TTC level (but not 1-day studies), as substances in this class are extremely toxic at low doses and often unsuitable for longer-duration testing due to their toxicity. This approach is explained in sections 3.3 (“Derivation of the Pre-validation EDT TTC Levels”) and 4.6.3 (“Derivation of the Finalized TTCs Based on the Combined EDT DB”).

iii) Regarding statistical significance, one peer reviewer inquired whether FDA reevaluated the statistical significance of reported effects in the studies. FDA notes that we reviewed safety studies for over 3,100 compounds, many of which had multiple studies. Reevaluating the statistical significance of all observations in these 10,000+ studies was beyond the project's resources. This information was added to section 2.1 (“Creation of the Original (Pre-validation) EDT Chemistry. Toxicology, and Metabolism Database (EDT DB)”) and to section 4.3.7 (“Additional Processing of the Data in the External Validation DB”).

iv) For adverse effects evaluation, as discussed in FDA's response to a similar comment (see #4 above), we incorporated current knowledge on whether effects are adverse and their relevance to humans when assigning a NOAEL or NOEL during the review of all available studies for a specific substance, particularly older evaluations.

7. **Improvement Suggestions**:

a) Add headings to section 2.2 to improve readability.

FDA agrees that adding sub-headings to section 2.2 will enhance readability. Additionally, the information in this section has been reorganized for improved clarity and flow. The newly created subsections are as follows:

2.2.1 Criteria for Data Collection
2.2.2 Derivation of Duration Adjusted NELs
2.2.3 Adjustment of NELs Based on Dosing Schedule and Test Article Purity

2.2.4 Consideration of Sex- and Species-specific Effects and Metabolism When Establishing NELs

b) Clarify the treatment of offspring vs. parental NELs.

FDA acknowledges that section 2.2 of the peer review document may not have clearly explained how we derive duration-adjusted NO(A)ELs from reproductive and/or developmental toxicity studies. To enhance clarity, we have updated the original text of the peer review document. The revised paragraph now states: “In reproductive and/or developmental studies, systemic parental NO(A)EL, reproductive NO(A)EL, and developmental NO(A)EL values are normally provided (or LO(A)EL if no NO(A)EL can be established). For these studies, we assign the lowest NO(A)EL as the overall study NO(A)EL. If the systemic parental or reproductive NO(A)ELs are chosen as the overall study NO(A)EL, we apply duration adjustment factors of either 3 or 10 to generate chronic NO(A)ELs, with the specific factor selected based on the study length. However, if the developmental NO(A)EL is lower than that for either or both parents and the reproductive NO(A)EL, we select the developmental NO(A)EL without adjusting for study duration. The reason for not applying a duration adjustment factor to developmental NO(A)ELs is that adverse developmental effects arise from in utero exposure within a predefined and relatively short time frame; they are not the result of chronic exposure to the test article by the fetus. This approach aligns with the ICH Harmonised Guideline (ICH, 2016), which specifies a duration adjustment factor (AF) of 1 for “reproductive studies in which the whole period of organogenesis is covered.” Additionally, the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) Technical Report No. 110 (ECETOC, 2010) indicates that for developmental toxicity, “an AF [adjustment factor] for exposure duration is not necessary provided that the experimental exposure includes the entire period of gestation, parturition, and the first four days of postnatal life.” Consequently, an adjustment factor for exposure duration is generally not required, resulting in an “informed” AF of 1.” Therefore, FDA believes that our approach for deriving duration-adjusted NO(A)ELs for reproductive and/or developmental studies is reasonable and consistent with established practices.

c) Discuss the rationale behind not distinguishing between genotoxic and non-genotoxic compounds in the criteria.

Long term chronic and cancer studies are often the most robust source of toxicological information for a chemical. Therefore, as long as toxicological studies yielded an overall NO(A)EL for which neither carcinogenic nor noncarcinogenic effects were observed for a carcinogenic substance, we included the substance in the DB and used its NO(A)EL to calculate its class TTC, regardless of whether it was a non-genotoxic or genotoxic carcinogen. We note that there are non-threshold mode of actions and other factors, such as short-term study durations (less than 1 or 2 years) that might have contributed to the fact that a NO(A)EL could be derived for some carcinogens.

FDA would like to emphasize that while we included these compounds in our database and designed EDT questions to capture them, it is ultimately up to each user, including regulatory agencies, to determine whether to apply the EDT for nongenotoxic and/or genotoxic carcinogens based on specific regulatory frameworks and program areas.

While these studies and substances were included in the EDT database, use of the EDT is not intended to replace assessment of genotoxicity or cancer risk assessment.

d) Explain what is meant by "uncatalyzed metabolism".
Uncatalyzed metabolism refers to biochemical reactions that occur without the assistance of enzymes. These reactions typically include hydrolysis (the breakdown of compounds by the addition of water, which can occur without enzyme involvement) and non-enzymatic conjugation (reactions where small molecules (like glutathione) may react with electrophiles without specific enzyme catalysis). To enhance clarity, this was added to section 2.2.4 ("Considerations of Sex- and Species-specific Effects and Metabolism When Establishing NELs") of the EDT peer review document as a footnote.

e) In section 2.2, FDA mentions studies with "limited reporting". Explain whether "limited" refers to the number of animals, whether the observed effects were adverse, or something else.
"Limited reporting" refers to the amount of data and information provided regarding study observations. In some cases, only a brief summary of the study results was available, lacking detailed information on the findings. This clarifying statement was added to section 2.2.1 ("Criteria for Data Collection") of the peer review document as a footnote.

f) In section 2.2, FDA talks about species differences. A peer reviewer suggested the following edit to this section: "For example, for aliphatic, alicyclic, or aromatic ketones or hydrocarbons of sufficient molecular weight and lipophilicity that cause α_{2u}-globulin-type nephropathy, an endpoint not relevant to humans, and observed exclusively in male rats, we used the toxicological data (e.g., NEL and LEL values) for female rats only for inclusion in the EDT DB."
This input prompted FDA to update the original text of "For example, we used the toxicological data (e.g., NEL and LEL values) for inclusion in the EDT DB for female rats only for aliphatic, alicyclic, or aromatic ketones or hydrocarbons of sufficient molecular weight and lipophilicity that cause α_{2u}-globulin-type nephropathy, an endpoint not relevant to humans, and observed exclusively in male rats." to "For instance, we included toxicological data (i.e., NEL and LEL values) for female rats only in the EDT DB for aliphatic, alicyclic, or aromatic ketones or hydrocarbons that possess sufficient molecular weight and lipophilicity, which cause α_{2u}-globulin-type nephropathy—a non-relevant endpoint to humans that is observed exclusively in

male rats."

References (in order of appearance in FDA's responses):

International Council for Harmonisation (ICH) of Technical Requirements for Pharmaceuticals for Human Use (2016). ICH Harmonised Guideline Impurities: Guideline for Residual Solvents Q3C(R6). Retrieved from https://database.ich.org/sites/default/files/Q3C-R6_Guideline_ErrorCorrection_2019_0410_0.pdf

European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) (2010). *Technical Report No. 110 Guidance on Assessment Factors to Derive a DNEL.* Retrieved from http://www.ecetoc.org/wp-content/uploads/2014/08/ECETOC-TR-110-Guidance-on-assessment-factors-to-derive-a-DNEL.pdf

Nohmi T. (2018). Thresholds of Genotoxic and Non-Genotoxic Carcinogens. *Toxicological research*, *34*(4), 281–290. https://doi.org/10.5487/TR.2018.34.4.281

Fukushima, S., Gi, M., Kakehashi, A., & Wanibuchi, H. (2014). Threshold of Genotoxic Carcinogens: Consideration from No-Effect Doses for Cancer-Related Markers in Chemical Carcinogenesis. *Mutagenesis, 29*(6).

Kakehashi, A., Fukushima, S., Wei, M., & Wanibuchi, H. (2014). Threshold in Carcinogenicity of Genotoxic Carcinogens. *Journal of Carcinogenesis & Mutagenesis, S3*, 6-12. http://dx.doi.org/10.4172/2157-2518.S3-006

Hengstler, J. G., Bogdanffy, M. S., Bolt, H. M., & Oesch, F. (2003). Challenging dogma: thresholds for genotoxic carcinogens? The case of vinyl acetate. *Annual review of pharmacology and toxicology*, *43*, 485–520. https://doi.org/10.1146/annurev.pharmtox.43.100901.140219

Zito R. (2001). Low doses and thresholds in genotoxicity: from theories to experiments. *Journal of experimental & clinical cancer research*, *20*(3), 315–325.

Müller, L., & Kasper, P. (2000). Human biological relevance and the use of threshold-arguments in regulatory genotoxicity assessment: experience with pharmaceuticals. *Mutation research*, *464*(1), 19–34. https://doi.org/10.1016/s1383-5718(99)00163-1

Lutz, W. K., & Kopp-Schneider, A. (1999). Threshold dose response for tumor induction by genotoxic carcinogens modeled via cell-cycle delay. *Toxicological sciences*, *49*(1), 110–115. https://doi.org/10.1093/toxsci/49.1.110

Nohmi, T., & Tsuzuki, T. (2016). Possible mechanisms underlying genotoxic thresholds: DNA repair and translesion DNA synthesis. In *Thresholds of Genotoxic Carcinogens* (pp. 49-66). Academic Press.

Kaina, B., Thomas, A. D., & Hengstler, J. G. (2015). Do Carcinogens Have a Threshold Dose? Pro and Contra. In *Regulatory Toxicology* (pp. 397-406). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-35374-1

Question 11: FDA used various factors based on study duration to derive duration adjusted no-effect-levels (NELs) to estimate chronic NELs. Has FDA provided adequate

information and/or data to support its opinion that these duration adjustment factors are adequate to derive chronic NELs? If you generally agree, are there any exceptions in which these factors might be problematic to the derivation of duration adjusted NELs?

Summary of general impressions:

The peer reviewers generally appreciated the thoroughness of FDA's approach to deriving duration-adjusted chronic no-effect levels (NELs). They highlighted the need for transparency and clarity in documenting the adjustment factors and processes used. The following recommendations for improvement were provided:

1. **Transparency**: Clearly document the adjustment factors and the processes for applying them.
2. **Non-Oral Studies**: Provide additional information on conversion factors used for non-oral studies. The analysis of the impact of non-oral studies would be welcomed.
3. **Consistency in Detail**: Ensure consistent detail is provided on selecting duration adjustment factors employed for the derivation of duration adjusted NELs for both the pre-validation ("original") EDT DB and the External Validation DB.
4. **Bias in Single-Dose Studies**: Evaluate and address potential biases as a result of including single-dose studies.
5. **Reproductive and/or Developmental Studies**: Provide additional clarification on how the NELs were adjusted for duration for reproductive and/or developmental studies and the reasoning behind the factors employed.
6. **Improve Clarity**: Reference to the "Original" EDT in section 2 is confusing. Clarify what is meant by "Original".
7. **Provide Explanation**: Explain how the situation was handled where a NEL for the shorter duration study was lower than that for the chronic study.

FDA response:

We sincerely thank the peer reviewers for their insightful feedback and constructive suggestions regarding our methodology and the clarity of our documentation. Their input is invaluable in enhancing the robustness and transparency of our EDT development process.

Regarding **transparency**: In response to charge question 11, some peer reviewers emphasized the need for clear documentation of the duration adjustment factors used to derive chronic NO(A)ELs. This concern was also mentioned in question 10, where FDA provided a comprehensive response and updated the peer review document accordingly. For further details, please refer to FDA's response in question 10.

Regarding **non-oral studies**: One peer reviewer requested additional information on the conversion factors used for non-oral studies and welcomed an analysis of the impact of allowing non-oral studies in the combined EDT DB. FDA clarifies that the same duration adjustment factors applied to oral studies were also used for non-oral studies when deriving duration-adjusted NO(A)ELs. Non-oral NO(A)ELs were similarly adjusted based on dosing schedules and test item purity. As mentioned in a previous response regarding non-oral studies, less than 4% of the studies in the combined EDT DB involved parenteral administration. Parenteral studies were included only when oral studies were either unavailable or deemed unsuitable, such as when they involved only one or two animals per dose or when substances could not be administered orally due to their physicochemical properties. Given that parenteral studies constitute a small fraction of the dataset, FDA anticipates that they will have minimal to no impact on the class TTCs.

Upon extensive evaluation, FDA identified that the only congeneric group where the lack of oral studies and the prevalence of parenteral studies might lead to potential "overclassification" is prostaglandins. As detailed in section 4.4.3.35 of the peer review document, the available data indicate that prostaglandins possess very high biological activities. Unfortunately, most toxicological data are derived from intravenous studies, and publicly available oral toxicological data are quite limited. This limited availability often stems from extensive first pass metabolism, which typically precludes oral use of prostaglandins in drug formulation. Nevertheless, many prostaglandins are active when administered orally. Therefore, based on the scarce publicly available oral preclinical toxicological data, prostaglandins are classified into Class VI, erring on the side of caution until further oral safety data can refine their classification.

Regarding ensuring **consistent detail** is provided on selecting duration adjustment factors employed for the derivation of duration adjusted NELs for both the pre-validation (“original”) EDT DB and the External Validation DB: this issue was previously raised and fully addressed by FDA. For the FDA’s complete response, please refer to question 10.

In response to concerns about **potential biases from** including **single-dose studies**, FDA emphasizes that the majority of studies in the combined EDT DB were conducted at multiple dose levels, as stipulated by authoritative guidelines such as those from OECD. There are a total of 126 substances in the Combined EDT DB that had a NO(A)EL and were either single dose studies or only one dose level was known, and it was unclear whether the study was single dose or multi-dose (to err on the side of caution, the latter studies were assumed to be single-dose studies). (Note that single-dose studies that did not produce a NEL were not counted as they could not be used for TTC calculations.) Of these 126 substances, 28, 24, 38, 23, 10, and 3 were Class I, II, III, IV, V, and VI, respectively. The effect of these studies on the class TTCs was examined by recalculating the 5th percentile NELs (which were used to calculate the class TTCs). These newly calculated values (highlighted grey in the table below and in non-Italic) were compared to

the 5th percentile NELs calculated considering all studies, including single dose studies (highlighted blue and in *Italic* in the table below).

EDT Class	I	II	III	IV	V	VI
5th percentile NEL (µmol/kg bw/day)	*213.9*	*24.10*	*6.236*	*1.038*	*0.01705*	*1.628E-04*
5th percentile NEL – no single-dose studies (µmol/kg bw/day)	231.5	27.38	7.043	1.100	0.01701	1.546E-04
%Change*	-7.6%	-12%	-11%	-5.6%	0.2%	5.3%

*% Change: This is the % change in the value of the 5th percentile NELs, used for calculating TTCs, when single dose studies are included in the TTC calculation.

For Classes I, II, III, and IV, when used for the 5th percentile NEL calculations, single-dose studies have somewhat lowered the 5th percentile NELs by 7.6%, 12%, 11%, and 5.6%; that is, they made the TTCs for the compounds sorted into these classes more conservative. This is unsurprising as some compounds (often flavoring substances) are tested at a low level (much lower than the expected true NEL) to ensure that the study would produce a NEL. In summary, the use of single-dose studies for Classes I, II, III, and IV makes the TTCs of these classes more protective. For Class V, the effect of single-dose studies is very small. For Class VI, including single-dose studies for the calculations slightly (by 5.3% or an absolute value of about 0.0820) increased the 5th percentile NEL of this class.

In response to the request for FDA to **clarify what is meant by "Original"**: FDA recognizes that the use of "original EDT" in section 2 may be confusing, as it refers to the pre-validation EDT. To enhance clarity, we have changed every instance of "original" to "original (pre-validation)" throughout the EDT peer review document, not just in section 2. Additionally, we replaced "originally" with "prior to validation" where applicable.

One of the peer reviewers asked FDA to **clarify how situations were handled when a NEL from a shorter duration study was lower than that from a chronic study**. FDA acknowledges that in some instances, shorter duration studies for the same substance produced lower NELs than those derived from longer studies. We identified three main reasons for this:

1. Shorter duration studies often assess additional toxicological endpoints, providing more thorough testing that may be cost-prohibitive or unnecessary in chronic studies. As a result, these shorter studies can yield lower NELs compared to chronic studies for the same substance.

2. In some shorter duration studies, effects may be observed, but the authors may be uncertain whether these effects are adaptive or indicative of potential adverse effects. To err on the side of caution, effects deemed uncertain are often classified as adverse. If a chronic study is conducted later, it may reveal that the effect observed in the shorter study was actually adaptive, leading to a lower original NEL (as assigned by the study authors) from the shorter study compared to the chronic NEL.
3. Chronic studies may not include tests for reproductive or developmental toxicity or teratogenicity, even though these endpoints can often produce the lowest NO(A)ELs. Most reproductive and developmental toxicity studies have shorter durations than chronic studies.

In response to the **request for FDA to clarify how it addressed cases where the No-Effect Level (NEL) for a shorter-duration study was lower than that for a chronic study, and to explain how NELs were adjusted for duration in reproductive and/or developmental studies, including the rationale behind the factors used**: FDA previously provided a detailed explanation addressing these points. Peer reviewers also raised this question in their comments on question 10, to which FDA responded comprehensively. For complete details, please refer to FDA's response to question 10.

In summary, we encountered cases where a shorter duration study yielded a lower NO(A)EL than a chronic study for the same substance. In such cases, we assigned the study with the lowest NOAEL as the best representative study for the substance to ensure that the most sensitive endpoint was considered.

Question 12: Based on Figure 2 and all other information provided, in your opinion, does the EDT better resolve the differing toxic potentials of chemicals with broad structural variation compared to the CDT? Please explain why or why not.

Summary of general impressions:

The peer reviewers generally agree that the EDT offers improved resolution of the differing toxic potentials of chemicals compared to the CDT. They emphasize that the updated classes in the EDT and the incorporation of contemporary scientific knowledge increase confidence in its use for TTC analysis. However, they express a need for additional information, data, and clarification.

Specific Suggestions and Requests for Additional Information:

1. **Clarification on Substance Count**: Explain the difference between the total number of substances in each class and those used for TTC calculations, including reasons for any exclusions.
2. **Support for Class VI**: Acknowledge potential criticism regarding the low number of substances in Class VI, while reinforcing support for this classification due to its

representation of uniquely toxic compounds.

3. **Additional Analytics for Tables 2 and 3**: Include ranges of molecular weights (MWs) and NELs in Tables 2 and 3, along with 5th and 95th percentiles to provide a more realistic view. Provide the MW range covered by the CDT (FDA: peer reviewer meant: Munro DB).
4. **Data Request**: Comment on how many orders of magnitude the Cramer Decision Tree (CDT) DB (FDA: the Munro DB) data spans to highlight the additional NELs covered by the EDT.
5. **MW cutoff**: Please clarify if there is a cutoff for MW before a large complex organic compound is excluded from the applicability domain of the EDT.
6. **Use of Molar Units**: Explain the rationale for using molar units instead of mg and how this choice affects data distribution.
7. **Selection of EDT Classes**: Clarify the criteria used to determine the number of EDT Classes.
8. **Comparison of NEL Units**: Suggest recalculating CDT TTCs in µg/kg bw/day and converting NELs in the CDT (i.e., Munro) DB to mmol/kg bw/day for a comparative analysis with EDT TTCs.
9. **Choice of Default EDT Class**: Request information on how the default EDT Class was selected.
10. **Comparison with Recent TTC Improvements**: Suggest including comparisons of EDT curves against recent TTC improvements that incorporate additional groups.

FDA response:

FDA would like to thank the peer reviewers for their valuable input regarding the evaluation of the EDT's ability to better differentiate the toxic potentials of chemicals than the CDT.

For clarity, ease of presentation, and due to length of responses, the specific suggestions and comments above will be restated below, with FDA's responses added in blue starting on a new line.

1. **Clarification on Substance Count**: Explain the difference between the total number of substances in each class and those used for TTC calculations, including reasons for any exclusions.
   The original (pre-validation) EDT DB includes 1,900 substances. Some studies produced only LOAELs and no NOAELs; therefore, while these data contributed to formulating the EDT structure-based questions and determining the best class assignments for their respective congeneric groups, they could not be used for class TTC calculations due to the absence of NOAELs. As noted in the first paragraph of section 3.3 of the peer review document, the pre-validation EDT DB, which contains 1,628 NO(A)ELs, was sufficiently robust for its intended purpose, which is why FDA opted not to derive additional NOAELs from LOAELs. Additionally, some substances had representative studies with a duration of less than 84 days. As previously mentioned, NO(A)ELs from studies shorter than 84 days were excluded from TTC calculations for Classes I-V. To clarify the discrepancy between the total number of substances in each class and those used for TTC calculations, we have added the

following note to Table 2 ("The pre-validation EDT TTCs") in section 3.4: The discrepancy between the total number of substances in each class and those used for TTC calculations arises because some studies provided only LOAELs and no NOAELs, which prevented their inclusion in the calculations. Additionally, NO(A)ELs from studies shorter than 84 days were excluded from TTC calculations for Classes I-V. The same note was also added to Table 8 ("The finalized (post-validation) EDT TTCs") in section 4.6.3.

2. **Support for Class VI**: Acknowledge potential criticism regarding the low number of substances in Class VI, while reinforcing support for this classification due to its representation of uniquely toxic compounds.
   While the original (pre-validation) EDT database contained only 52 Class VI substances, of which 46 could be used for the Class VI TTC calculation, the combined EDT database—upon which the finalized TTCs are based—now includes 91 Class VI substances, more than three times the number used in the original Munro Class II TTC. For the Class VI TTC calculations, NO(A)ELs for 70 of these 91 substances were utilized, which is more than double the number used for the original Munro Class II TTC calculation.

   Class VI aims to capture the most toxic substances that exist. Due to their extreme toxicity and often limited commercial applicability, Class VI substances are not typically tested in oral studies other than acute. Consequently, despite a thorough screening of a wide range of toxicological databases, we were unable to identify additional Class VI substances with subacute, subchronic, chronic, or reproductive and developmental toxicity studies for inclusion in the combined EDT database of 3,141 substances.

   Given the extremely low EDT Class VI TTC value of 0.00053 µg/kg bw/day, we believe this threshold is sufficiently protective for any substances assigned to Class VI. This assumption is based on the fact that this TTC is lower than any other published TTC values and represents only one-fifth of the genotoxicity carcinogenicity threshold of 0.15 µg/person/day (0.0025 µg/kg bw/day) (Kroes et al., 2005). Therefore, FDA anticipates that the Class VI TTC level will effectively protect all substances classified within EDT Class VI.

   The above paragraphs with minor edits were added to section 4.6.3 "Derivation of the Finalized TTCs Based on the Combined EDT DB" of the peer review document in support of the EDT Class VI TTC.
3. **Additional Analytics for Tables 2 and 3**: Include ranges of molecular weights (MWs) and NELs in Tables 2 and 3, along with 5th and 95th percentiles to provide a more realistic view. Provide the MW range covered by the CDT (i.e., Munro DB).
   Rather than adding the aforementioned data to Tables 2 and 3, which pertains only to the pre-validation EDT database, this information has been included in Table 8 of section 4.6.3, which presents data for the combined EDT database used to establish the finalized (post-validation) EDT TTCs. This approach ensures completeness.

   Unfortunately, the FDA is unable to compare the MW range of the Munro DB with that of the combined EDT DB, as the published Munro DB does not include MW information

for the substances it contains (Munro et al., 1996). The MW range of substances in the combined EDT DB is 30.03-2285.61. The NO(A)EL range for studies in the Munro database is 0.005-6883 mg/kg bw/day, while the combined EDT database spans 0.000001-9000 mg/kg bw/day. Thus, the combined EDT database encompasses a broader range of NO(A)ELs compared to the Munro database.

4. **Data Request**: Comment on how many orders of magnitude the CDT (i.e., Munro) DB data spans to highlight the additional NELs covered by the EDT.
   As stated above, the NO(A)EL range for studies in the Munro database is 0.005-6883 mg/kg bw/day, while the combined EDT database spans 0.000001-9000 mg/kg bw/day. Thus, the combined EDT database encompasses a broader range of NO(A)ELs compared to the Munro database. Thus, the NO(A)ELs in the Munro DB span 6 orders of magnitude, while the NO(A)ELs in the combined EDT DB span nearly 10 orders of magnitude when expressed in mg/kg bw/day. Consequently, the combined EDT DB encompasses a much broader range of NO(A)ELs, indicating that it includes substances with a wider array of toxic potentials compared to the Munro DB.
5. **MW cutoff**: Please clarify if there is a cutoff for MW before a large complex organic compound is excluded from the applicability domain of the EDT.
   There are no MW restrictions for excluding organic compounds from the applicability domain of the EDT. While the largest MW in the combined EDT DB is 2285.61 Da, 95% of the compounds in this database have a MW of 651.20 Da or lower.
6. **Use of Molar Units**: Explain the rationale for using molar units instead of mg and how this choice affects data distribution.
   As stated in the last paragraph of section 2.1 of the peer review document, consistent with recommendations from various publications, we represent toxic potency using study duration adjusted NELs expressed in mmol/kg bw/day (Escher et al., 2010; Tluczkiewicz et al., 2011). This approach allows for comparisons of NELs between substances based on the number of molecules present at the NEL. For example, 0.1 mmol, or 7 mg of acetone (molecular weight (MW)=70 mg/mmol), contains the same number of molecules as 0.1 mmol, or 111 mg of ciguatoxin (MW=1111 mg/mmol). When comparing the toxic potency of different substances, a weight-to-weight comparison must consider the differences in their molecular weights. Therefore, mole-based NEL adjustments provide a scientifically robust approach for developing structural classes of *relative* toxicity. Moreover, mole-based NELs can improve sensitivity in detecting potential toxicity, particularly for substances with very low mass but high biological activity, ensuring that even low concentrations of highly potent substances are adequately evaluated.

   Using the units of mmol/kg bw/day for the EDT NELs did not affect the results depicted in Figure 2, as both the Munro NO(A)EL data and the EDT NO(A)EL data used for this figure were expressed in the same units.
7. **Selection of EDT Classes**: Clarify the criteria used to determine the number of EDT Classes.
   As mentioned earlier, the NO(A)ELs in the Munro DB span 6 orders of magnitude, while those in the combined EDT DB span nearly 10 orders of magnitude when expressed in mg/kg bw/day. When converted to mmol/kg bw/day, the NO(A)ELs in the combined EDT DB span an impressive 11 orders of magnitude. This indicates that the

substances in the combined EDT DB have NO(A)ELs that cover nearly double the range of those in the Munro DB. Consequently, FDA concluded that it was essential to double the number of classes used by Munro to adequately account for the expanded range observed in the combined EDT DB compared to the Munro DB. The FDA revised section 3.3 of the peer review document to clarify the reasoning for doubling the number of classes compared to the Cramer/Munro classes.

8. **Comparison of NEL Units**: Suggest recalculating Cramer Decision Tree (CDT) TTCs in µg/kg bw/day and converting NELs in the CDT (i.e., Munro) DB to mmol/kg bw/day for a comparative analysis with EDT TTCs.
   The TTC values currently in the published literature are expressed as weight/person/day (e.g., µg/person/day) or weight/body weight/day (e.g., µg/kg bw/day). Therefore, FDA provided a comparison of the CDT TTCs and the EDT TTCs, expressed in µg/kg bw/day, in the table below. Generally, Cramer Class I substances encompass EDT Classes I and II (though Cramer Class I is overprotective for substances captured by EDT Class I), Cramer Class II corresponds to EDT Class III, and Cramer Class III aligns with EDT Class IV. However, Cramer Class III, as originally designed, does not adequately protect against substances categorized in EDT Classes V and VI.

EDT Class	**I**	**II**	**III**	**IV**	**V**	**VI**
Finalized EDT TTC (µg/kg bw/d)	385	45	12	2.9	0.052	0.00053
CDT Class	I		II	III		
Cramer TTC (µg/kg bw/day) (Munro et al., 1996)	30		9	1.5		

9. **Choice of Default EDT Class**: Request information on how the default EDT Class was selected.
   The primary default class for the CDT is CDT Class III. Substances lacking specific CDT questions addressing their structural features are assigned to this class by default. We have significantly expanded the number of structure-based questions in the EDT and reduced the number of substances without questions addressing their structural features, minimizing the reliance on default classifications.

   For the EDT, the main default class is EDT Class IV, provided the compound does not contain any highly toxic structural features associated with EDT Classes V and VI. The FDA considers this approach reasonable for two reasons: first, before assigning a compound to EDT Class IV, we verify that it does not possess features warranting placement in EDT Classes V or VI; second, as indicated in the table above, the TTC for CDT Class III is comparable to that of EDT Class IV.
10. **Comparison with Recent TTC Improvements**: Suggest including comparisons of EDT curves against recent TTC improvements that incorporate additional groups.

The FDA acknowledges that numerous updates to the original CDT and Munro TTCs have been proposed over time. For example, TTCs have been established for specific congeneric groups, such as organophosphates (where a single TTC value is used despite the wide toxicity range within this group), or TTC value recalculations following the removal of certain congeneric groups (e.g., organophosphates, organohalogens, and others) from the three main CDT TTC classes.

The FDA aimed to update the original CDT to reflect the current state of science and to encompass all relevant groups of substances for which group-specific TTCs were proposed. As such, this type of analysis would not provide meaningful insights. For example, organophosphates and organohalogens are distributed across all six EDT classes rather than assigned to a single class. So, comparing congeneric group specific TTCs is not possible.

References:

Kroes, R., Kleiner, J., & Renwick, A. (2005). The threshold of toxicological concern concept in risk assessment. *Toxicological sciences*, *86*(2), 226-230.

Munro, I. C., Ford, R. A., Kennepohl, E., & Sprenger, J. G. (1996). Correlation of structural class with no-observed-effect levels: a proposal for establishing a threshold of concern. *Food and Chemical Toxicology*, *34*(9), 829-867.

Tluczkiewicz, I., Buist, H. E., Martin, M. T., Mangelsdorf, I., & Escher, S. E. (2011). Improvement of the Cramer classification for oral exposure using the database TTC RepDose–a strategy description. *Regulatory Toxicology and Pharmacology*, *61*(3), 340-350.

Question 13: Has FDA clearly explained the source of the validation DB and how the data was verified pre-validation? If not, what additional information should we provide?

Summary of general impressions:

The peer reviewers generally found that the FDA provided a clear explanation of the source of the validation database, specifically identifying it as the US Environmental Protection Agency's ToxVal database. They noted that the processing and verification methods appeared robust. However, there were a few areas where additional information and clarification were requested to enhance transparency and comprehensiveness.

List of Requested Improvements and Clarifications

1. **Provide Reference and URL**: Include the URL or reference for the ToxVal database, along with version numbers and dates of data collection, to allow for tracking updates.
2. **Clarify Data Discrepancies**: Report on discrepancies between the original study reports and the ToxVal database, along with explanations for these inconsistencies.
3. **Detail the NOAEL Determination Process**: Ensure that the process for determining the NOAEL is fully described, clarifying any ambiguity around the use of "our own

judgement," and confirm that all decisions are documented in the database.
4. **Specify Minimum Study Duration**: Indicate the minimum number of days a study had to be conducted to be included in the validation database.
5. **Overview of Databases Used**: Provide a clear overview of the databases utilized for the EDT and the External Validation Database, including the number of chemicals extracted from each and any overlaps. Include tables and figures to help guide the reader in understanding the data sources and relationships.

FDA response:

FDA thanks the peer reviewers for their valuable feedback, which will enhance the clarity and rigor of our validation database by improving data transparency, addressing discrepancies, and providing a comprehensive overview of our methodologies.

Regarding the request to provide the URL or reference for the ToxVal database, along with version numbers and dates of data collection, to allow for tracking updates: The URL for the ToxVal DB is https://comptox.epa.gov/dashboard/chemical-lists/TOXVAL_V5. This URL was added to section 4.2 of the EDT peer review document.

The FDA requested assistance from the U.S. EPA in identifying substances for validating the pre-validation EDT. On February 8, 2021, the EPA provided a database of over 20,000 lines of toxicological data in Excel format to support this effort. According to Dr. Antony Williams of the U.S. EPA, these data were sourced from the ToxVal database developed by Dr. Richard Judson over several years. Dr. Williams explained that he filtered Dr. Judson's latest file to meet FDA criteria, focusing on compounds with defined structures, oral studies only, and those longer than acute duration. He also mapped these data to relevant information like SMILES codes and molecular weight. In his email, Dr. Williams mentioned that the dataset came from the ToxVal February 3, 2021 data update, and while much of it is available on the dashboard, FDA is receiving the latest version. Therefore, the FDA is unable to provide a version number. A summary of this information has been added to section 4.2 ("Creation of the External Validation Database") in the peer review document.

Regarding providing details on discrepancies between original study reports and the ToxVal DB: The FDA verified the accuracy of all toxicological study data—such as the identity of test items, species, duration, dose levels, and NO(A)ELs—harvested from the ToxVal database to be used for the external validation.

We discovered that some studies listing a specific substance as the test article were actually conducted with a different test article (a read-across substance), particularly in cases referencing ECHA. This discrepancy arises because ECHA often employs a significant number of read-across studies to present safety data for specific substances. For instance, for the entries for strontium 2-ethylhexanoate (CAS 2457-02-5), the actual test item was strontium chloride (a read-across substance of strontium 2-ethylhexanoate), which falls outside the EDT's applicability domain as it is an inorganic substance (hence was not added to the DB). A thorough examination of the ECHA site and other resources

revealed no studies listing strontium 2-ethylhexanoate as the test article, leading to its removal from the EDT database. Whenever the listed test item did not match the actual test item, the incorrect name was removed, and the correct test item name and identifiers were added to associate it with the study as long as the “correct test item” was in the applicability domain of the EDT. If the true (“correct”) test item was already present in the pre-validation or validation databases, the study entry was deleted to avoid duplicate entries for the same substance.

In addition to ensuring that each toxicological study was correctly associated with its respective test chemical, we verified that the correct NO(A)EL value was listed for each study by reviewing original study reports, when available, and other pertinent documents. Our search extended to opinions and risk assessments from various authoritative bodies, including the FDA, EPA (Human Health Risk Assessment documents, IRIS, RED, HPV), CalEPA, ECHA, EFSA, EMA, JECFA, and JMPR. Notably, we found discrepancies in the NO(A)EL values reported by these organizations. For example, in the 24-month rat study on Chlorpyrifos-methyl (CAS 5598-13-0) (Barna-Lloyd et al., 1991), the EPA and JMPR reported a NOAEL of 1 mg/kg bw/day (EPA, 2015; JMPR, 2009), while the EFSA reported 0.1 mg/kg bw/day (EFSA, 2019). In such cases, we evaluated all available data from all sources to determine the most appropriate NO(A)EL for each study in the external validation set. For more information on this, please see FDA’s response to question 9.

Additionally, some NOAELs listed were not overall NOAELs but only carcinogenic NOAELs, potentially overlooking noncarcinogenic adverse effects. This issue was particularly evident for chronic-duration studies referencing the NTP. We also noted instances of the same study being listed multiple times with varying NOELs, depending on the endpoint or generation examined (in reproductive studies). For instance, the NOAEL for cyclopentanethiol (CAS 1679-07-8) was initially recorded as 0.06 mg/kg bw/day in the DB received from the EPA, referencing the EFSA Scientific Opinion on Flavouring Group Evaluation 91 (FGE.91). However, upon reviewing the EFSA publication, we found a NOEL of 0.56 mg/kg bw/day based on an unpublished study (Morgareidge, K., Oser, B.L., 1970b). A thorough review of the scientific literature and authoritative evaluations confirmed the NOEL of 0.56 mg/kg bw/day (e.g., JECFA, Safety Evaluation of Certain Food Additives and Contaminants, WHO Food Additive Series: 44. Simple Aliphatic and Aromatic Sulfides and Thiols). Consequently, we updated the NOEL to 0.56 from 0.06 in the validation DB to reflect the correct value.

Moreover, for some compounds, the NOAEL initially assigned to a specific study by a specific authoritative body changed over time during the rereview of the same study by the same authoritative body. For example, for Spiromesifen (CAS 283594-90-1) the same 2-generation study (Eiben et al., 2002) was listed 6 times in the Excel sheet received from EPA; four times with a NOAEL of 2.2. mg/kg bw/day, once with a NOAEL of 3.3 mg/kg bw/day, and once with a NOAEL of 4.6 mg/kg bw/day. The 2007 EPA memorandum titled “Spiromesifen. Human Health Risk Assessment for a section 3 Registration on Beans.” placed the male parental systemic NOAEL at 2.2. mg/kg bw/day and the offspring NOAEL at 4.6 mg/kg bw/day (EPA, 2007). The 2020 EPA document titled “Spiromesifen. Draft

Human Health Risk Assessment in Support of Registration Review" states that the results of this study were re-evaluated, and the systemic NOAEL was "bumped up to" 8.8 from 2.2 mg/kg bw/day for parental males (EPA, 2020). Also, EPA changed the offspring NOAEL from 4.6 mg/kg bw/day (EPA, 2007) to 3.8 mg/kg bw/day (EPA, 2020) to correct an erroneous assignment in the 2007 document. FDA notes that according to EFSA, the offspring NOAEL for this study is 3.3. mg/kg bw/day (EFSA, 2007 & 2012) confounding the final NOAEL selection. Consequently, there was some confusion regarding the lowest NOAEL of this study. Nonetheless, based on the data and information available, whatever the correct NOAEL of the above study might be, it is not expected to be lower than 3.3 mg/kg bw/day. In addition to the Eiben et al. (2002) study with the questionable NOAEL, a mouse carcinogenicity study with a NOAEL of 3.3 mg/kg bw/day is available. The NOAEL of this study is not under question and does not exceed any possible NOAELs of the Eiben et al. (2002) study. Therefore, FDA decided to choose the mouse carcinogenicity study as the representative study for this substance. We note that this study was not included in the original external validation set and was, therefore, entered into the external validation DB by FDA.

In summary, all data received from EPA were verified and corrections were made when justified.

References:

Barna-Lloyd, T.; Szabo, J.; Davis, N. (1991) Chlorpyrifos- Methyl (Reldan Insecticide): Chronic Dietary Toxicity/Onco- genicity Study in Rats: Lab Project Number: K-046193-031. Unpublished study prepared by Dow Chemical Co., Lake Jackson Research Center. MRID 42269001. Available from 1) EPA, 2015. Chlorpyriphos-Methyl: Human Health Draft Risk Assessment (ORA) for Registration Review at https://www.regulations.gov/document/EPA-HQ-OPP-2010-0119-0020, 2) JMPR (2009) Chlorpyrifos-methyl Monograph at https://apps.who.int/pesticide-residues-jmpr-database/Document/101, and 3) EFSA (2019) Updated statement on the available outcomes of the human health assessment in the context of the pesticides peer review of the active substance chlorpyrifos-methyl at https://www.efsa.europa.eu/en/efsajournal/pub/5908

EPA (2015). Chlorpyriphos-Methyl: Human Health Draft Risk Assessment (ORA) for Registration Review. Available from Regulations.gov at https://www.regulations.gov/document/EPA-HQ-OPP-2010-0119-0020

EFSA (2019). Updated statement on the available outcomes of the human health assessment in the context of the pesticides peer review of the active substance chlorpyrifos-methyl. *EFSA Journal, 17*(11), e05908.

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF). (2011). Scientific Opinion on Flavouring Group Evaluation 91, Revision 1 (FGE. 91Rev1): Consideration of simple aliphatic and aromatic sulphides and thiols evaluated by JECFA (53rd and 68th meetings) structurally related to aliphatic and alicyclic mono-, di-, tri-, and polysulphides with or without additional oxygenated functional groups evaluated by EFSA in FGE. 08Rev3 (2011). EFSA Journal, 9(12), 2459.

Morgareidge K and Oser BL, 1970b. 90-Day feeding studies in rats with cyclopentanethiol (31025). Food and Drug Research Laboratories, Inc. Lab. no. 0032. August 24, 1970. Unpublished report submitted by EFFA to FLAVIS Secretariat. Available from EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF). (2011). Scientific Opinion on Flavouring Group Evaluation 91, Revision 1 (FGE. 91Rev1).

JECFA, Safety Evaluation of Certain Food Additives and Contaminants, WHO Food Additive Series: 44. Simple Aliphatic and Aromatic Sulfides and Thiols at https://inchem.org/documents/jecfa/jecmono/v44jec09.htm

Eiben, R.; Bach, U.; Rinke, M. (2002) BSN 2060 Two-Generation Study in Wistar Rats: Lab Project Number: PH 31775: T507099: G200100. Unpublished study prepared by Bayer AG. 976 p. MRID 45819619. Available from EPA, 2017.

EPA (2007). Spiromesifen. Human Health Risk Assessment for a Section 3 Registration on Beans. Available from Regulations.gov at https://www.regulations.gov/document/EPA-HQ-OPP-2007-0331-0004

EPA (2020). Spiromesifen. Draft Human Health Risk Assessment in Support of Registration Review. Available from Regulations.gov at https://www.regulations.gov/document/EPA-HQ-OPP-2014-0263-0021

EFSA (2007). Conclusion regarding the peer review of the pesticide risk assessment of the active substance spiromesifen. *EFSA Journal*, 5(7), 105r.

EFSA (2012). Conclusion on the peer review of the pesticide risk assessment of the active substance spiromesifen. *EFSA Journal*, 10(10):2879

Regarding the details of the NOAEL determination process: For all studies, we reviewed evaluations from the study authors and various authoritative bodies, as discussed earlier (see FDA's responses to question 9). When all evaluations agreed on the NO(A)EL for a study, the FDA adopted that value. However, there were often discrepancies among authoritative bodies regarding the NO(A)EL for the same study. In such cases, we evaluated all studies for that substance from all available sources, considering the reasoning behind the NO(A)EL selections made by authoritative bodies and study authors. In these instances, the FDA used its best judgment, employing our most current understanding of what constitutes an adverse effect and its relevance to human health risk assessment when selecting the study NO(A)ELs. For example, to determine whether an effect is adverse, non-adverse, adaptive, or an artifact, the FDA consulted various sources, including Pandiri et al. (2017) [Toxicologic Pathology, 45(1), 238-247]. We also note that sometimes study reports, evaluations, or summaries lacked sufficient data to determine whether an effect was adverse, adaptive, or an artifact. When we could not determine whether an effect was adverse or not, to err on the side of caution, we assumed that it was adverse (this sentence was added to section 2.1.("Creation of the Original (Pre-validation) EDT Chemistry, Toxicity, and Metabolism Database (EDT DB)) of the peer review document. Additionally, differing opinions exist in the scientific literature regarding the relevance of certain adverse effects observed in laboratory animals to human health risk assessment. It is beyond the scope of this project to delve into the best practices for determining whether an adverse effect is truly adverse and relevant to humans. Finally, we note that in our combined EDT database, we identified instances where various

authoritative bodies disagreed on the NO(A)EL, or where the value was reassessed over time to a different figure.

Regarding the study duration requirements for inclusion in the validation set: We included only non-acute studies in the External Validation Database. Similar to the original (pre-validation) EDT database, our goal was to collect chronic studies; however, these were often either unavailable or, as discussed earlier in response to question 11, shorter-duration studies yielded lower NO(A)ELs than chronic studies. For the pre-validation and the finalized (post-validation) EDT TTC calculations for Classes I-V, a study's NO(A)EL must be derived from a study with a minimum duration of 84 days and the NO(A)EL is only used for TTC calculation after the derivation of the chronic NO(A)EL using adjustment factors. This requirement is detailed in FDA's response to question 11 and in sections 3.3 ("Derivation of the Pre-validation EDT TTC Levels") and 4.6.3 ("Derivation of the Finalized TTCs Based on the Combined EDT Database") of the EDT peer review document.

Regarding the request to provide a clear overview of the databases utilized for the EDT and the External Validation Database, including the number of chemicals extracted from each and any overlaps: A similar request was made in the peer reviewers' comments for question 9. For the FDA's complete response, please refer to the answer provided for question 9.

Question 14: Has FDA clearly laid out how the validation DB received from EPA was processed to enable its use for the external validation of the EDT? If not, please explain why not.

Summary of general impressions:

The peer reviewers generally found that the FDA provided clear and detailed information on the processing and verification of the validation database. They appreciated the thoroughness of section 4.3, which outlined the steps taken to eliminate duplicate substances and select appropriate data. However, some of the reviewers noted areas needing clarification or additional information.

Requests for Clarification or Improvement

1. **Cross-Referencing**: Clarify how chemicals already in the original (pre-validation) EDT DB were identified and removed from the external validation DB (e.g., using CASRN or SMILES).
2. **SMILES Consistency**: Specify which type of SMILES code was used and recommend adopting a standard approach, such as canonical SMILES or InChI/InChIKeys, to ensure consistency and accuracy in identifying duplicates.
3. **SMILES Canonicalization**: Confirm whether the SMILES from both the EDT DB and the validation DB were standardized using the same software prior to comparison.
4. **Counterions**: Provide a list of counterions removed from the validation DB and clarify if different salt forms were run through the EDT workflow before removal.

5. **Different ADIs/RfDs**: Explain the approach taken when different agencies provided varying Acceptable Daily Intakes (ADIs) or Reference Doses (RfDs), including whether the lowest was chosen or if judgment was used to determine the most appropriate value, along with the factors considered.
6. **Study Selection Criteria**: Describe the rationale behind selecting certain studies as more appropriate, including the criteria used (e.g., study length, adverse effects, lower NEL).
7. **IUPAC Names**: Confirm if IUPAC names were recorded where available.
8. **Applicability Domain**: Suggest investigating the chemical space of the EDT DB and assessing whether the validation DB meets this, potentially using techniques like Principal Component Analysis.
9. **Read-Across Data**: Clarify the treatment of substances where the true test item turned out to be a read-across substance, especially considering the challenges associated with ECHA's database.
10. **Typographical Errors**: Correct identified typos in section 4.3.1.

These suggestions aim to enhance the clarity, rigor, and comprehensiveness of the FDA's validation process.

FDA response:

We would like to express our sincere gratitude to the peer reviewers for their insightful feedback. Their inputs help us identify areas for clarification and improvement. The peer reviewers' suggestions on cross-referencing, data consistency, selection criteria, and applicability domain will help ensure the robustness and transparency of our methodology.

For clarity, ease of presentation, and due to length of FDA's responses, the specific suggestions and comments above will be restated below, with FDA's responses added in blue starting on a new line.

1. **Cross-Referencing**: Clarify how chemicals already in the original (pre-validation) EDT DB were identified and removed from the external validation DB (e.g., using CASRN or SMILES).
   FDA has addressed this clarification request in response to question 9. For more details, please refer to FDA's response to that question.
2. **SMILES Consistency**: Specify which type of SMILES code was used and recommend adopting a standard approach, such as canonical SMILES or InChI/InChIKeys, to ensure consistency and accuracy in identifying duplicates.
   Unfortunately, our combined EDT DB contains various types of SMILES codes, which has reduced their effectiveness for identifying duplicates. However, the FDA also employed CAS numbers, common names, and IUPAC names to assist in this process. Additionally, as outlined in our response to question 9, we manually reviewed all substances captured at the same question, sub-question, or sub-sub-question for duplicates. We are confident that we effectively eliminated duplicates from the combined EDT DB.
3. **SMILES Canonicalization**: Confirm whether the SMILES from both the EDT DB and

the validation DB were standardized using the same software prior to comparison.
As noted above, we did not standardize SMILES codes and allowed the presence of various types of SMILES codes in our database.

4. **Counterions**: Provide a list of counterions removed from the validation DB and clarify if different salt forms were run through the EDT workflow before removal.
   Depending on the EDT questions, users are instructed to disregard specific (mostly) non-organic counterions. The handling of counterions is detailed at the beginning of post-validation EDT questions 1, 2, and 3, with a final explanation and disposition provided in question 5 regarding all salts. The approach to counterions in these questions varies based on their toxic potential and whether they might influence the overall toxicity of the substance more than the organic component. For instance, sodium counterions are consistently disregarded in questions 1, 2, 3, and 5, as they are unlikely to be the primary drivers of toxicity. In contrast, strontium counterions are considered due to their toxic potential and their possible role in the compound's toxicity. The FDA notes that the handling of counterions will be automated in the EDT software currently in development, eliminating the need for user input.
5. **Different ADIs/RfDs**: Explain the approach taken when different agencies provided varying Acceptable Daily Intakes (ADIs) or Reference Doses (RfDs), including whether the lowest was chosen or if judgment was used to determine the most appropriate value, along with the factors considered.
   The TTC values provided in the EDT are derived from NO(A)EL values not ADIs or RfDs. The NO(A)EL serves as the initial benchmark, representing the highest dose at which no adverse effects were observed in studies. The RfD is calculated by dividing the NO(A)EL (or sometimes the LOAEL) by various uncertainty factors that account for inter- and intraspecies differences, as well as data limitations. Similarly, the ADI is derived in a comparable manner, focusing on acceptable daily intake levels. By applying these uncertainty factors, both the ADI and RfD provide conservative estimates of safe exposure levels over a lifetime, minimizing potential risks.

   In this context, the TTCs function similarly to the ADI and RfD.

   To establish sufficiently conservative predictive safe exposure levels (i.e., TTCs), we employed only NO(A)ELs. We applied a factor of 100 to account for inter- and intraspecies differences and used the class low 5th percentile NO(A)ELs to further ensure conservativeness and address additional uncertainties. In summary, during the development of the EDT and the derivation of the EDT TTCs, we exclusively used NO(A)ELs, without incorporating ADIs or RfDs. The use of *only* NO(A)ELs (i.e., NELs) for TTC calculation is discussed in the peer review document in sections 3.3 (“Derivation of the Pre-validation EDT TTC Levels) and 4.6.3 (“Derivation of the Finalized TTCs Based on the Combined EDT DB). To avoid any confusion, we added “Please note that only NELs were used for the calculation of the TTCs and no ADIs or RfDs.” to section 3.3.
6. **Study Selection Criteria**: Describe the rationale behind selecting certain studies as more appropriate, including the criteria used (e.g., study length, adverse effects, lower NEL).
   In its response to question 9, the FDA detailed the criteria for including studies in its

toxicological database. The corresponding section in the EDT peer review document was reorganized and expanded in response to this request. Additionally, in its response to question 11, the FDA explained how it addressed situations where a NEL from a shorter-duration study was lower than that from a chronic study, specifically regarding which study was chosen as the representative one. Therefore, the FDA will not reiterate this information here but will provide a brief overview.

We reviewed all available safety data for each substance to identify the best representative study on which to establish predicted chronic safe intake levels (i.e., TTCs). While we preferred to use data from chronic studies, many substances either lacked chronic studies or had shorter studies that produced lower NO(A)ELs (as noted in question 11). When both NOELs and NOAELs were available for the same substance, we selected the study with the lowest NOAEL, considering the relevance of observed adverse effects in laboratory animals to human health (as discussed in detail in FDA's response to question 10).

In summary, we considered multiple factors when selecting the best representative study—a process that is standard at the FDA for establishing acceptable daily intakes (ADIs) and reference doses (RfDs). During the selection of the best representative study, various publications, such as WHO 2008; WHO, 2018; FDA Redbook; EPA (2002), and Pandiri et al., 2007, were used to guide us.

References:

WHO (2008). Environmental Health Criteria 240. Principles and methods for the risk assessment of chemicals in food. Available at https://www.who.int/publications/i/item/9789241572408

WHO (2018). IPCS harmonization project document; no. 11. Guidance document on evaluating and expressing uncertainty in hazard characterization, 2nd edition. Available at https://www.who.int/publications/i/item/9789241513548

FDA (2020). Redbook. Guidance for the Industry and Other Stakeholders. Toxicological Principles for the Safety Assessment of Food Ingredients. Available at https://www.fda.gov/media/79074/download

EPA (2002). A review of the reference dose and reference concentration process. Available at https://www.epa.gov/sites/default/files/2014-12/documents/rfd-final.pdf

Pandiri, A. R., Kerlin, R. L., Mann, P. C., Everds, N. E., Sharma, A. K., Myers, L. P., & Steinbach, T. J. (2017). Is it adverse, nonadverse, adaptive, or artifact?. *Toxicologic pathology*, 45(1), 238-247.

7. **IUPAC Names**: Confirm if IUPAC names were recorded where available.
   IUPAC names are included in the combined EDT DB for all substances.
8. **Applicability Domain**: Suggest investigating the chemical space of the EDT DB and assessing whether the validation DB meets this, potentially using techniques like Principal Component Analysis.
   The FDA found the comment slightly confusing and assumes that by "EDT DB," the

peer reviewer was referring to the Original (pre-validation) EDT DB. As noted in the EDT peer review document, the pre-validation database contained 1,900 unique substances. For example, sodium, potassium, calcium, and some other forms of the same organic substance are considered one substance because these inorganic counterions are not expected to drive the toxicity of the organic counterion.

When creating the external validation database, our objective was not to target compounds within a specific chemical space; rather, we aimed to include all compounds within the applicability domain of the EDT for which non-acute oral studies in laboratory animals are publicly available. We sought to maximize the number of substances with broad structural diversity to ensure that the EDT is applicable across a wide range of chemicals with various uses. To accomplish this, we enlisted the help of the EPA, which was compiling a database of all publicly available toxicological studies (see FDA's response to question 13 for details).

Looking ahead, we plan to expand the combined EDT database and update the EDT questions based on newly available data, further enhancing its applicability domain.

Regarding the chemical space of the EDT DB: as it was already addressed elsewhere (see FDA's responses at question 3), the FDA will not revisit it in this section.

9. **Read-Across Data**: Clarify the treatment of substances where the true test item turned out to be a read-across substance, especially considering the challenges associated with ECHA's database.
   As stated in FDA's response for question 13, when QC-ing the external validation DB, we discovered that some studies listing a specific substance as the test article were actually conducted with a different test article (a read-across substance), particularly in cases referencing ECHA. This discrepancy arises because ECHA often employs a significant number of read-across studies to present safety data for specific substances. For instance, for the entries for strontium 2-ethylhexanoate (CAS 2457-02-5) the correct test item was strontium chloride (a read-across substance of strontium 2-ethylhexanoate), which falls outside the EDT's applicability domain as it is an inorganic substance. A thorough examination of the ECHA site and other resources revealed no studies listing strontium 2-ethylhexanoate as the test article, leading to its removal from the database. Whenever the listed test item did not match the actual test item, the incorrect name was removed, and the correct test item name and identifiers were added to associate it with the study. If the true test item was already present in the pre-validation or validation databases, the study entry was deleted to avoid duplicate entries for the same substance.
10. **Typographical Errors**: Correct identified typos in section 4.3.1.
    FDA acknowledges the inconsistent use of "counter ion" and "counterion" within the EDT peer review document. While both terms appear in chemistry, "counterion" (as a single word) is more widely accepted in scientific literature. Since it is preferable to use "counterion" when referring to the ion that balances the charge of another ion in a compound, FDA has changed every instance of "counter ion" to "counterion" throughout the EDT peer review document.

Question 15: Has FDA provided adequate information and/or data to show that the validation DB was processed appropriately for its intended use? If not, what additional information should we provide?

Summary of general impressions:

The peer reviewers generally expressed a mix of satisfaction and constructive criticism regarding the selection criteria for assigning the best representative toxicological study in the EDT DB. There was acknowledgment that FDA has provided sufficient information to demonstrate that the validation database was processed appropriately, with references to specific sections that detail additional processing.

Key points raised for addressing are as follows:

1. **Overview of Compounds**: Reviewers noted the absence of an overview detailing the types of compounds in the pre-validation EDT and External Validation Database, such as pesticides, food flavorings, and pharmacologically active substances. This information is crucial for evaluating the EDT's intended use across different sectors and its applicability to persistent chemicals.
2. **Clarity of Validation Terms**: Reviewers highlighted the need for a clearer definition of "validation," as its interpretation can vary. They suggested elaborating on the intended purpose of validation and what it means for the EDT TTCs to be "protective." Clarification on how much lower the TTCs are compared to the NO(A)EL was also requested.
3. **Prediction vs. Evaluation**: Concerns were raised regarding the statement that the EDT will “accurately predict the chronic oral toxicity.” Reviewers suggested that the purpose of TTC should not be to predict toxicity but rather to evaluate safety.

Overall, while there is confidence in the processing of data, reviewers called for improvements in defining key terms and providing additional context about the types of compounds included in the databases.

FDA response:

FDA thanks the peer reviewers for their valuable feedback on the selection criteria for representative toxicological studies in the EDT DB. Their insights on the need for clarity regarding compound types and validation definitions will help us enhance the transparency of the EDT and broaden the readers’ understanding.

Reviewers highlighted the lack of an overview regarding the types of compounds included in the original (pre-validation) and external validation databases, such as pesticides, food flavorings, and pharmacologically active substances. The FDA aimed at developing a decision tree capable of handling compounds with broad structural variation across various domains. Consequently, both our original (pre-validation) database and the external validation database feature compounds from diverse sources, including, but not limited to,

those naturally occurring in food (both safe compounds and natural toxins), food additives, food contact materials, pesticides, common industrial solvents, cosmetic ingredients, excipients in pharmaceuticals, and active pharmaceutical ingredients, as well as extractables and leachables. Many compounds in our databases have multiple applications. For example, benzyl alcohol serves as a solvent in pharmaceuticals and industrial processes, a flavoring agent in food, a preservative and fragrance fixative in cosmetics, an antimicrobial agent, and a laboratory reagent for various chemical analyses. Its broad applications span the food, cosmetics, pharmaceuticals, and industrial manufacturing sectors. This is true for numerous other compounds found in the combined EDT database. Therefore, including all potential applications and/or sectors for each compound is difficult and time and resource intensive. However, the FDA would like to remind future users of the EDT that each regulatory agency and its respective programs must determine how they will utilize the EDT and which compounds within its applicability domain are subject to its evaluation. Agencies may choose to limit the EDT's applicability domain based on their own rules, laws, and program requirements.

Reviewers highlighted the need for a clearer definition of "validation," as its interpretation can vary. They suggested elaborating on the intended purpose of validation. In response to this comment, FDA notes that we developed the EDT to assess the chronic oral toxic potential of a diverse range of compounds and to predict presumptively safe intake levels (i.e., to provide TTC levels). After creating the pre-validation EDT, we aimed to evaluate its performance and determine its suitability for this purpose. Specifically, we sought to ensure that it can accurately classify compounds into six distinct classes of relative chronic oral toxic potential and predict presumptively safe intake levels that genuinely offer protection against adverse effects.

The process we undertook to evaluate the EDT's performance is referred to as validation. Validation involves systematic testing and assessment to confirm that the EDT functions as intended. This included verifying that the classification system effectively differentiates compounds based on their toxic potential and that the predicted presumptively safe intake levels are reliable and protective for human health. By conducting this external, independent validation, we aimed to establish confidence in the EDT's ability to inform regulatory decisions and ensure safety across various applications.

One of the peer reviewers requested clarification on i) what it means for the EDT TTCs to be "protective" and ii) how much lower the TTCs are compared to the NO(A)EL. The FDA notes, that for a class TTC to be protective for a substance, it indicates that no adverse toxicological effects are anticipated at exposure levels at or below the substance's class TTC. In other words, the TTC serves as a sufficiently conservative predicted safe (protective) intake level, ensuring that adverse effects are only expected to occur at dose levels exceeding the substance's class TTC.

In general, a class TTC is significantly lower than the NO(A)ELs of substances within that class. This difference arises from our calculation method for class TTCs. As detailed in the peer review document, we examined all NO(A)ELs within the specific class and identified the low 5th percentile NO(A)EL, meaning that 95% of substances in that class had a

NO(A)EL higher than this value. We then applied a factor of 100 to account for inter- and intra-species differences. Consequently, class TTCs are much lower than the NO(A)ELs for substances in that class. While we recognize that this calculation is conservative, such conservativeness ensures high levels of protection for all substances within their classes. Furthermore, this approach aligns with the calculation methods for other presumptively safe intake levels, such as acceptable daily intake (ADI), where factors like 10x10 are applied to the NO(A)EL to address inter- and intra-species differences.

Concerns were raised regarding the statement that the EDT will “accurately predict the chronic oral toxicity.” Reviewers suggested that the purpose of TTC should not be to predict toxicity but rather to evaluate safety. FDA acknowledges that the EDT was not designed to predict chronic oral toxicity; rather, it classifies compounds into six classes based on their relative chronic oral toxic potential and predicts safe intake levels (i.e., TTC level). We agree that the phrase “accurately predict the chronic oral toxicity” should be revised to “accurately sorts compounds based on/according to their relative chronic oral toxic potential into six classes and provides presumptively safe intake levels” throughout the peer review document, and the necessary edits have been made. We also agree that the EDT serves as a valuable tool to assist FDA and other users in safety evaluations.

Phase II:

Question 16: Some of the pre-validation EDT questions were updated, and some new sub- and sub-sub-questions were created based on the validation results. Has FDA provided adequate information to justify all updates? If not, which changes/updates were not fully justified and what information should we provide to justify them?

Summary of general impressions:

The peer reviewers generally expressed positive feedback regarding the updates to the pre-validation EDT questions, noting that the changes were well explained and justified. They commended FDA for the extensive work completed during the validation process and acknowledged the importance of these updates in enhancing the EDT's usability for toxicological evaluations. However, they also provided specific suggestions for further improvements and clarifications.

Key points raised for addressing are as follows:

1. **Appendix Updates**: Integrate and update Appendix 1 with all changes made to the pre-validation EDT questions, as outlined in section 4.4 and tracked in section 4.5.4.
2. **Read-Across Methodology**:
   a) Elaborate on the principles and key criteria for the read-across approach.
   b) Compare available software programs (e.g., EPA's GenRA and ECHA's OECD Toolbox) to enhance transparency in the EDT's read-across methodology.

3. **Bioavailability Considerations**: Consider incorporating broader ADME understanding to improve predictions of systemic exposure, potentially using commercially available software (e.g., ACD Percepta).
4. **Clarifications on Specific Questions**:
   a) **Q6a**: Confirm whether data from the EDT DB or references were used to support the change.
   b) **Q7**: Address potential typos related to references to Q6b(i) and Q6b(ii).
   c) **Q7g**: Add an explanation for the clarifications made to this question.
   d) **Q7 Assignments**: Provide an explanation for the reassignment of chemicals answering "yes" at Q7g(iv).
   e) **Q14 Assignments**: Clarify the rationale for moving chemicals related to epoxides substituted by or fused to a polyaromatic ring in Q14b(ii).
   f) Address the assignment of polyepoxides, which were initially assigned to Class V instead of being moved to Q33.
5. **General Comments**: Provide further insights on ongoing updates and the implications for the future development of the EDT.

FDA response:

FDA would like to extend our sincere thanks to the peer reviewers for their valuable insights and constructive feedback on the EDT updates/validation. Their input has significantly aided FDA to enhance the clarity of our methodologies and ensuring the robustness of the validation.

One of the peer reviewers suggested that FDA integrate and update Appendix 1 with all changes made to the pre-validation EDT questions, as outlined in section 4.4 and tracked in section 4.5.4 (now section 4.5.5). FDA did not update Appendix 1 as all changes were provided in great detail in section 4.4 and clearly tracked in section 4.5.5 and because Appendix 1 was intended only for the pre-validation EDT. However, depending on resources, FDA may publish a comprehensive scientific rationale for each post-validation EDT question, potentially as a series of journal articles, which will serve as a valuable resource for those interested in structure-toxicity relationships.

Regarding the read-across methodology, FDA notes that most questions, sub-question, and sub-sub-questions are designed to capture compounds that are structurally, metabolically, and thus toxicologically similar. When a data-poor substance is evaluated using the EDT, data-rich substances in the combined EDT DB classified under the same question, sub-question, or sub-sub-question can be utilized as read-across substances. Alternatively, both a data-poor and a data-rich substance can be run through the EDT to determine if they are classified under the same question, sub-question, or sub-sub-question. FDA acknowledges that the paper version of the EDT requires expert input from the user to confirm that the read-across substance is a suitable analog for the data-poor substance. As mentioned elsewhere in this document, the EDT software is currently under

development. This software tool will automate the read-across process (eliminating the need for user input) and also provide structural similarity scores between the query compound and its analog. Until the EDT software is developed, we are unable to provide a comparison of the available software programs (e.g., EPA's GenRA and the OECD Toolbox) with the EDT software's read-across methodology at this time. Once the EDT software is completed, FDA will make it publicly available along with documentation to ensure transparency regarding its functionalities.

Regarding the suggestion that FDA consider incorporating broader ADME understanding to improve predictions of systemic exposure, potentially using commercially available software (e.g., ACD Percepta): We aimed to integrate broad ADME considerations into the EDT questions to ensure that the EDT effectively predicts the relative chronic oral toxic potential of a wide range of compounds, and we utilized commercially available software to assist in this process. In the future, we plan to update the EDT questions to reflect the latest scientific advancements and to further incorporate an even broader understanding of ADME.

Regarding the request for clarification for updating EDT Q6a): yes, toxicological data from the combined EDT DB were used to update Q6a).

Regarding the request to address potential typos related to references to Q6b(i) and Q6b(ii): the typos were corrected.

One of the peer reviewers requested an explanation for the clarifications made to Q7g). The updates to Q7g) during the external validation did not alter the types of compounds it was originally intended to capture. Instead, we added straightforward clarification statements to address misclassifications and confusion experienced by the validation chemists.[9] We recognize that the pre-validation version of Q7g) was somewhat ambiguous.

One of the peer reviewers requested an explanation for the reassignment of chemicals that answered "yes" at Q7g(iv). In the pre-validation EDT, compounds in this category were classified as Class IV, while in the post-validation EDT, they are now placed in Class V. FDA unintentionally did not provide a rationale for this change. This reassignment was based on a thorough review of the toxicological data for these compounds. The compounds in this sub-sub-question are primarily toxic due to the reactivity of the halogen atoms (F, Cl, Br, I) attached to the carbon atoms bearing ether oxygens, indicating a high potential for toxicity.

For example, chloro(methoxy)methane (CAS 107-30-2) produced a Lowest Effect Level (LEL) of 450 µg/kg bw/day, with no No Effect Level (NEL) identified (Laskin et al., 1975).

[9] As stated in section 4.4.1. ("The External Validation of the EDT") of the peer review document, "The EDT was validated with the help of the finalized external validation DB and the independent (non-government, external) scientists (referred to as validation chemists in the text of this document). These validation chemists have expertise in organic chemistry, metabolism, and structure-toxicity relationships."

While the NEL is expected to be below this value, how far below is unknown. Considering that the 5th percentile NEL for Class IV (the basis of the Class TTC value) is approximately 288 µg/kg bw/day, the potential NEL for this compound is likely significantly lower than the Class IV 5th percentile NEL. The 5th percentile NEL for Class V is around 5.2 µg/kg bw/day. Therefore, Class V is the most appropriate/protective for this substance.

Other highly toxic and suspected carcinogenic substances captured at the same sub-sub-question and included in this classification are bromo(methoxy)methane (CAS 13057-17-5), iodo(methoxy)methane (CAS 13057-19-7), (chloromethoxy)ethane (CAS 3188-13-4), and (bromomethoxy)ethane (CAS 53588-92-4). Some halogenated ethers are recognized for their neurotoxic effects; for instance, (chloromethoxy)ethane is known to be both a neurotoxin and hepatotoxin (see https://pubchem.ncbi.nlm.nih.gov/compound/18523#section=Toxicity).

Reference:

Laskin, S., Drew, R. T., Cappiello, V., Kuschner, M., & Nelson, N. (1975). Inhalation Carcinogenicity of Alpha Halo Ethers: II. Chronic Inhalation Studies With Chloromethyl Methyl Ether. *Archives of Environmental Health: An International Journal*, 30(2), 70-72.

The above rational is now included in section 4.4.3 “Justifications and Scientific Basis for Updating the EDT”.

Regarding the rationale for moving chemicals related to epoxides substituted by or fused to a polyaromatic ring from Q14b(ii)) to Q33: It’s important to clarify that not all epoxides were/are included in Q14 of the pre-/post-validation EDT, and this decision was intentional. FDA notes that this did not change when the pre- and post-validation EDT is compared. Some epoxides were/are specifically addressed by Q14 of the pre-/post-validation EDT, while others—along with their non-epoxide metabolic precursors and metabolites—were/are included in Q33 because their polyaromatic structural features warrant inclusion within the aromatic block of questions (Q33-47). In summary, we believe the epoxides covered in Q33 are more closely related to the non-epoxides in Q33 than to the epoxides addressed in Q14. Therefore, not all epoxides were/are classified under Q14 by the pre- and post-validation EDT.

One of the peer reviewers noted “The pre-validation EDT assigned polyepoxides to Class V, rather than moving them onto Q33.” unlike the post-validation EDT. FDA notes that this statement is not correct. Both the pre- and the post validation Q14 assign polyepoxides to Class V except those that are addressed at Q33.

Regarding the request for further insights on ongoing updates and their implications for the future development of the EDT: Depending on available resources, FDA plans to periodically update the EDT to ensure it reflects the latest scientific knowledge and advancements. We will also consider public input in these future developments. All updates and the rationale behind them will likely be communicated through journal

publications and/or updates on FDA's EDT website. As mentioned earlier, the EDT software is currently in development and is expected to be publicly available in 2026.

Question 17: Was the validation adequate to show that the EDT is suitable for the classification of compounds in its applicability domain according to their toxic potentials? If not, describe what type of validation would be needed.

Summary of general impressions:

Overall, the validation exercise was seen as useful. Some reviewers expressed that FDA did not adequately define the applicability domain of the EDT. There is a consensus that clearer definitions and visual representations of the applicability domains for each class would enhance user understanding. Reviewers emphasized the importance of defining the criteria for the EDT database, including the intended uses of substances and the identification of naturally occurring compounds.

Requests for Clarification and Action:

1. **Applicability Domain Definition**: Provide a clear definition of the applicability domain for each of the six classes.
2. **Chemical Classifications**: Include details about the types of chemistry associated with each class and the range of physicochemical properties (e.g., log P, molecular weight).
3. **Identification of Substances**: Clearly identify substances by their intended uses (e.g., food additives, plasticizers) and distinguish naturally occurring substances with significant bioactivity and potential toxicity.
4. **Bootstrapping Approach**: Implement a bootstrapping approach to compare the 5th percentile values of pre- and post-validation EDTs to demonstrate statistical differences or the lack of it.

FDA response:

FDA would like to express our sincere gratitude to the peer reviewers for their valuable insights and suggestions. Their feedback has greatly aided us in clarifying the applicability domain of the EDT and identifying areas needing further clarifications.

Regarding the applicability domain of the EDT and its six classes, the peer reviewers requested clarifications on various aspects, such as the molecular weight cutoff and the inclusion of organosilicons and endocrine disruptors within the applicability domain, and also asked FDA to provide a clear description of the applicability domain of the tool. Please refer to FDA's responses to these inquiries at questions #1, 3, 6, 8, 9, 12, and 14.

It is important to note that while the EDT has an established applicability domain, the six classes do not have specific individual domains. As detailed in our response to the peer reviewers' comments for question 3, the chemistries within each EDT class are highly diverse, encompassing compounds with broad structural variation. Consequently,

compounds with markedly different structures, metabolic pathways, toxicity endpoints, modes of action, and applications may be assigned to the same EDT class and thus share the same presumptively safe intake level (i.e., the same TTC level). For instance, Class I includes a wide range of presumptively safe compounds and those with low toxic potential, such as linear aliphatic and methyl-substituted primary alcohols, aldehydes, (di)carboxylic acids (with some exceptions), amino acids, various lactones, sugars, sugar alcohols and acids, bile acids and salts, benzoic acid, and related compounds, among many other chemical classes with varied applications.

Furthermore, even within the same chemical class, compounds can be assigned to different EDT classes. For example, linear or branched aliphatic hydrocarbons may fall into different classes based on their specific structures. While hexane is classified under Class IV at Q28d(i), substances with terminal double bonds conjugated with another double bond (i.e., terminal dienes) may be classified into either Class IV or III at Q28s(i) or Q28s(ii), respectively. Other compounds in this chemical class may even be categorized into Class I at Q9. Given this complexity, it is not feasible to describe EDT classes solely in terms of the chemical classes they encompass.

Regarding the request to include details about the types of chemistry associated with each class and the range of physicochemical properties, please see our response at question 3.

In response to the request that FDA identify substances by their intended uses (e.g., food additives, plasticizers) and distinguish naturally occurring substances with significant bioactivity and potential toxicity: For a complete response to this request, please refer to the second paragraph of FDA's reply to the comments from the peer reviewers regarding question 15.

And finally, regarding the suggestion that FDA implement a bootstrapping approach to compare the 5th percentile NEL values of pre- and post-validation EDTs to demonstrate statistical differences or the lack of it: The post-validation 5th percentile NEL was calculated using a significantly larger and expanded dataset compared to the pre-validation 5th percentile NEL, as well as a greatly enhanced tool (i.e., the post-validation EDT). Therefore, the FDA believes that performing a statistical analysis to determine whether the pre- and post-validation 5th percentile NELs are significantly different would not be particularly instructive.

Question 18: Regarding section 5 ("Conclusions"): Has FDA provided adequate information and/or data to support the conclusions found in this section? If not, what additional information should we provide?

Summary of general impressions:

The peer reviewers generally expressed positive feedback regarding the EDT, highlighting several key points:

1. **Adequate Support for Conclusions**: The report provides sufficient information to support the conclusions drawn in section 5 ("Conclusions"), particularly regarding the grouping of structurally or metabolically similar chemicals and the application of the TTC approach.
2. **Significant Milestone**: Reaching Phase 2 of the EDT project is acknowledged as a monumental achievement.
3. **Valuable Tool**: The EDT is seen as an invaluable tool for predicting toxic potentials and providing presumptively safe intake levels, with the potential to become an industry standard.
4. **Encouragement for Future Development**: The development of EDT software to automate read-across is welcomed.
5. **Periodic Review and Refinement**: Support for the notion that the EDT should undergo periodic reviews and refinements as more data become available.

Requests for Clarifications or Action:

1. **Clarification on ADME Data, Modeling Bioavailability, and Route-to route Extrapolation**: Provide more detailed information on the extent of ADME data considered in the analysis, as current references may not adequately support the conclusions. Offer insights into how the EDT can relate oral absorption data to systemic bioavailability and provide guidance on route-to-route extrapolation factors.
2. **Development of Read-Across**: Expand on the development of read-across in the report to provide more detailed guidance and support for this conclusion.
3. **Future Guidance for Read-Across Framework**: Clarify how the EDT can be effectively integrated into a broader read-across framework and provide future guidance on this aspect.

FDA response:

FDA thanks the peer reviewers for their insightful feedback on the EDT project; their input will be instrumental in refining our data and enhancing our methodologies, particularly regarding ADME data and read-across approaches. Their recognition of our progress and encouragement for future developments are greatly appreciated as we strive to improve chemical classification and safety assessments.

As discussed in FDA's response to the peer reviewers' comments to question 16, FDA aimed to integrate broad ADME considerations into the EDT questions to ensure that the EDT effectively predicts the relative chronic oral toxic potential of a wide range of compounds, and we utilized commercially available software to assist in this process. In the future, we plan to update the EDT questions to reflect the latest scientific advancements and to further incorporate an even broader understanding of ADME. Many EDT questions take into account metabolic precursors and toxic metabolites of hazardous compounds. Moreover, while this may not be immediately apparent to readers, the classification of substances and their corresponding questions often reflect the consideration of the oral bioavailability of the compounds in question.

FDA would like to reiterate that the EDT was specifically designed to predict *oral* toxic potential and presumptively safe *oral* intake levels. It is important to note that the toxicity of a compound can vary significantly depending on the route of exposure—oral, dermal, or inhalational. For instance, diacetyl, which is commonly used for its buttery flavor in popcorn, is generally regarded as safe for ingestion. However, inhaling diacetyl can lead to severe respiratory problems, including "popcorn lung" (bronchiolitis obliterans), which damages the airways. To enable the EDT to accurately assess chronic inhalational or dermal relative toxic potential and provide presumptively safe exposure levels, considerable additional work would be necessary, including changing some of the EDT questions, the class assignments after certain questions, and building inhalational and dermal toxicological databases to enable FDA to calculate inhalational and dermal TTCs. There are no 'simple' factors to assist users in extrapolating from oral to other routes of exposure.

For further details on read-across, please refer to FDA's responses to the peer reviewers' comments at question 16. Additionally, regarding the integration of the EDT into a broader read-across framework, FDA plans to offer more insights in the future, potentially through a guidance document.

Appendix 1 aims at providing a brief explanation of each EDT question. By no means are these explanations meant to be comprehensive. With that in mind, please respond to the following questions.

Question 19: Are all explanations clear and concise? If not, please identify the explanation by question number and elaborate as to how we can more clearly explain the question.

Summary of general impressions:

The peer reviewers generally found the explanations in Appendix 1 to be clear and correct, appreciating their potential value for users of the EDT. While some reviewers acknowledged the clarity of the explanations, they also noted areas where updates and further details could enhance understanding, particularly in the context of finalized questions.

Requested Clarifications and Updates:

1. Update Appendix 1 to reflect the finalized EDT questions, providing detailed descriptions of compounds and associated mechanisms of action and toxicity endpoints.
2. Clarify the basis for the terminal Question 28 and its sub-questions (i-v), specifically regarding how toxicity endpoints are ranked into Classes III, IV, or V, and whether weighting is applied to account for severity.
3. Consider reordering the explanations to align with the order of the questions, while being mindful of potential repetition that could affect conciseness.

FDA response:

FDA thanks the peer reviewers for their valuable input and comments. Their insights have been instrumental in identifying areas for clarification and enhancement, particularly regarding the explanations in Appendix 1.

Regarding updating Appendix 1: FDA responded to this suggestion at question 16. Please see our response there.

Regarding the clarifications requested for EDT Q28, FDA notes that Q28 is a terminal question consisting of 20 sub-questions, labeled from a) to t), with some having additional sub-sub-questions. The maximum number of sub-sub-questions is four, labeled from i) to iv). Therefore, FDA is uncertain about the following request for clarification from one peer reviewer: "It would be helpful to provide the basis for this terminal question, sub-questions i), ii), iii), iv), and v) in ranking these toxicity endpoints into Classes III, IV, or V. For example, is weighting applied to the endpoints to account for severity leading to different Class assignments?" Since there are no sub-questions marked i) to v) and no sub-question has 5 sub-sub-questions, FDA believes the peer reviewer may have intended to inquire about the basis for assigning compounds to various classes at Q28. The rationale for sorting compounds into different classes at Q28 is based on their varying toxic potentials, informed by toxicological and ADME data in the combined EDT DB, as well as our understanding of modes of their toxic action. This approach is consistent with other complex questions (e.g., Qs 3 and 6), which also categorize a diverse array of compounds into various EDT classes. Also, within the same sub-question when multiple sub-sub-questions are present to sort related compounds, if they are assigned to various classes, it is due to their differing toxic potentials.

Question 20: Should FDA add anything to these explanations to improve the reader's understanding of each question's rationale? If yes, please identify the explanation by question number and explain how we should revise. Please note that these explanations were designed to be concise and not all-encompassing.

Summary of general impressions:

The peer reviewers generally found Appendix 1 to be clear and effective in aiding the reader's understanding of each pre-validation EDT question's rationale. They appreciated the level of detail and the references provided, noting that the explanations are well-suited for a knowledgeable audience, such as toxicologists and risk assessors. Overall, they recognized the value of the appendix while suggesting enhancements to further improve clarity and usability.

Clarifications and Requests for Updates:

1. **Title Addition**: Add a title to each question in Appendix 1 that summarizes and

describes the individual EDT questions.

2. **Applicability Domain**: Summarize aspects of applicability domain in Appendix 1, along with exemplar compounds and associated data.
3. **Integration of Updates**: Integrate updates from section 4.4 and corresponding edits in section 4.5.4 into Appendix 1 for a fuller understanding.
4. **Reaction Mechanism Illustrations**: Include illustrative examples of the reaction mechanisms for questions related to chemical (de)toxification (e.g., Qs 7b(ii), 19b, 19d, 32a, 33, and 38).
5. **Compounds Count**: Indicate how many compounds in the database were classified according to each EDT question.
6. **Details on Weighting and Ranking**: Provide additional details on the weighting and ranking of toxicity endpoints in Question 28, which differentiates between Classes III, IV, and V.

FDA response:

FDA sincerely thanks the peer reviewers for their insightful feedback and thoughtful suggestions regarding Appendix 1 and the overall clarity of our document. Their input has been invaluable in identifying areas for improvement, such as enhancing the explanations and integrating updates, which will help us provide clearer guidance for users of the EDT.

Regarding the suggestion to add a title to each question in Appendix 1 that summarizes and describes the individual EDT questions: Many questions address a wide range of substances. For instance, EDT Q1 encompasses a diverse array of safe compounds and those with low toxic potential, including linear aliphatic and methyl-substituted primary alcohols, aldehydes, and (di)carboxylic acids (with some exceptions), amino acids and some of their derivatives, various lactones, sugars, sugar alcohols, bile acids, benzoic acid, and other chemical classes with varied applications. Q3 addresses close to a hundred different N and/or S containing functional groups and moieties. Therefore, implementing this request is neither feasible nor practical.

Regarding the request for FDA to summarize aspects of the applicability domain in Appendix 1, along with exemplar compounds and associated data: The applicability domain of the EDT is already outlined in section 1.5 of the EDT peer review document and further elaborated in various questions throughout this response document. Additionally, example compounds are provided after each question, sub-question, and sub-sub-question. The EDT database contains over 3,100 example compounds, each accompanied by basic toxicological data, references, and the relevant classification question. Therefore, we believe it is unnecessary to duplicate this information in Appendix 1. Furthermore, adding this data would significantly lengthen Appendix 1, potentially diminishing its overall usefulness. Resources allowing, FDA plans to update and expand Appendix 1 at a later date, as mentioned elsewhere.

Regarding the request to integrate updates from section 4.4 and corresponding edits in section 4.5.4 (now section 4.5.5) into Appendix 1 for a fuller understanding: This was already addressed at questions 16 and 19. Please see FDA's response at those questions.

Regarding the request for FDA to update Appendix 1 with illustrations of reaction mechanisms: due to time and resource constraints, this request will be considered for a future update of Appendix 1.

In response to the request for the number of compounds classified according to each EDT question: There are over 200 questions, sub-questions, and sub-sub-questions in the EDT. Compiling the number of substances from the combined EDT database, which contains over 3,100 compounds, for each question, sub-question, and sub-sub-question is time-consuming and we believe it offers limited, if any, additional insights. Furthermore, due to the cross-checking feature of the post-validation EDT, a substance may receive a "yes" response at multiple questions. However, for each of the 3,100+ compounds in the combined EDT database, FDA has documented the path taken through the EDT, including the final question, sub-question, and sub-sub-question where the compound was classified. Interested stakeholders can query the combined EDT database to find out how many and which substances are classified under specific questions, sub-questions, or sub-sub-questions.

Regarding the request that FDA provide additional details on the weighting and ranking of toxicity endpoints in Question 28, which differentiates between Classes III, IV, and V: please see FDA's response for the previous charge question.

Appendix 2 contains the combined, finalized EDT Chemistry, Toxicology and Metabolism DB on which the finalized TTCs were based.

Question 21: Are the set of chemicals in the database sufficient to cover the chemical domain of applicability described in the document? If not, please explain.

Summary of general impressions:

The peer reviewers acknowledged the significant achievement of the FDA in assembling a large and carefully curated database of toxicological information. While they recognized the strengths of the database, they expressed that assessing the chemical domain of applicability requires more detailed analytics, such as structural features and property ranges. There was consensus that, although Class VI has limited data points due to its high toxicity, its relevance and significance are acknowledged. The reviewers asked for some clarifications, information, and updates.

Requests for Updates, Information, and Clarifications:

1. Provide a breakdown of how many compounds are associated with each question and class assignment in the EDT.
2. Add molecular weight units to Column E of Appendix 2 (e.g., µg/kg bw/day or mmol/kg bw/day) and include a footnote or text in Column E to clarify how molecular weight is adjusted based on the number of subunits.
3. Add a column for the intended/approved use of each compound (e.g., direct food

additive, indirect food additive, plasticizer, etc.).
4. Include a column for the chemical congeneric group/class/family for each compound.
5. Add a column for critical toxicity endpoint(s) that the EDT class is based on.

FDA response:

FDA would like to extend its sincere gratitude for the peer reviewers' insightful feedback and comments.

The molecular weight unit of g/mol was added to Column E of Appendix 2.

Regarding the request that FDA provide a breakdown of how many compounds are associated with each question and class assignment in the EDT: The total number of compounds in the combined EDT DB within each EDT class is provided in section 4.6.3 of the peer review document. Regarding providing a breakdown of how many compounds are associated with each question, see FDA's response to peer review question 20. A note was added right under Table 8. ("The finalized (post validation) EDT TTCs") in section 4.6.3 of the peer review document to explain MW adjustment based on subunits. Moreover, an example adjustment based on the presence of two subunits was provided in a footnote.

Regarding the request that FDA add a column for the intended/approved use of each compound (e.g., direct food additive, indirect food additive, plasticizer, etc.): FDA has previously addressed similar inquiries in response to peer review question 15. Briefly, the combined EDT database includes, but is not limited to, compounds from a wide array of sources, such as naturally occurring substances in food (both safe and toxic), food additives (direct and indirect), food contact materials, pesticides, industrial solvents, cosmetic ingredients, pharmaceuticals as well as extractables and leachables. Many compounds have multiple applications. For example, benzyl alcohol serves as a solvent in pharmaceuticals and industrial processes, a flavoring agent in food, a preservative and fragrance fixative in cosmetics, an antimicrobial agent, and a laboratory reagent for various chemical analyses. Its broad applications span the food, cosmetics, pharmaceuticals, and industrial manufacturing sectors. This is true for numerous other compounds found in the combined EDT database. Also, each compound may have various approved uses within the same agency and/or across multiple regulatory agencies that can also vary from country to country. Given the diverse applications and varying regulatory approvals across different countries and agencies, it is not feasible to provide an exhaustive list all potential uses and the regulatory status for the over 3,100 compounds in the combined EDT database.

In response to the request for a column indicating the chemical congeneric group/class/family for each compound, FDA added this information to the EDT DB (see more on this at question 3).

In response to the request for a column indicating the critical toxicity endpoint(s) for each EDT class: For each substance in the combined EDT DB, such a column already exists. That is, for each substance the critical endpoint(s) of toxicity is provided. It's important to note that no critical toxicity endpoint is exclusive to a single class; for example, hepatotoxicity may appear across multiple classes. The distinction between classes lies in the strength or potential of the compound to cause that endpoint, rather than the endpoints themselves.

Overall question:

Question 22: Do you have any other comments or suggestions?

Summary of general impressions:

The peer reviewers expressed strong appreciation for the extensive work undertaken by the FDA in developing the EDT, acknowledging its complexity, detailed structure, and broad coverage across chemical classes. They noted the significant achievement of curating a large database of toxicological information and highlighted the EDT's potential to improve upon the original Cramer scheme. Reviewers emphasized the importance of clear communication and detailed explanations in future publications, as well as the necessity for robust software support to facilitate understanding and acceptance of the EDT. They encouraged the FDA to promote the EDT through various channels and to ensure ongoing engagement with the scientific and regulatory communities.

The development of the EDT software platform is seen by the peer reviewers as vital for aiding safety assessors in navigating the complexities of the EDT, ultimately promoting its acceptance within the global safety assessment community. The reviewers agree that a well-designed software tool will significantly enhance the utility and reliability of the EDT, making it a valuable resource for assessing chemical safety.

Requests for Clarifications and Updates:

1. **General Suggestions:**
   a) Include a clean slate approach for the software platform rather than viewing it as an update to the Cramer scheme.
   b) Consider linking the EDT to the data in the database in the software application and integrating analyses using ToxPrint fingerprints and investigating property and chemical structure space.
   c) Promote the EDT through workshops, conferences, and social media and provide public training for the EDT.
2. **Specific Clarifications and Updates:**
   a) Improve readability by using square brackets for additional context and placing sub-sub-questions on separate lines.
   b) Address a question regarding the applicability of certain exemptions.
   c) Provide clarification for Q3f(iii)).

d) Address concerns regarding the coverage of certain toxic substances, particularly in Classes V and VI, and clarify the applicability of the EDT to nanomaterials and radioisotopes.
e) Discuss the inclusion of naturally occurring toxins, the overall comprehensiveness of the toxin space covered, and the placement of yessotoxins into Class VI.
f) Determine the best place to provide a definition for the term “connector”.
g) Correct the typos listed, rephrase certain statements, and update some examples.

FDA response:

FDA would like to extend its sincere gratitude for the peer reviewers’ invaluable feedback on the EDT. Their insights have greatly assisted us in identifying areas for improvement and refinement, particularly regarding clarity, comprehensiveness, and the overall structure of our framework. The peer reviewers’ recognition of the extensive work put into developing the EDT and its database reinforces our commitment to advancing this important tool. We appreciate the peer reviewers’ suggestions for promoting the EDT and ensuring its effective integration into regulatory practices. Their comments will play a crucial role in enhancing the clarity and utility of our documentation and software support.

For clarity, ease of presentation, and due to length of FDA’s responses, the specific suggestions and comments above will be restated below, with FDA’s responses added in blue starting on a new line.

1. **General Suggestions:**
   a) Include a clean slate approach for the software platform rather than viewing it as an update to the Cramer scheme.
   FDA is implementing a clean slate approach for the EDT software platform and is not planning to simply update existing software tools for Cramer classifications.
   b) Consider linking the EDT to the data in the database in the software application and integrating analyses using ToxPrint fingerprints and investigating property and chemical structure space.
   The FDA plans to integrate the EDT database into the software platform to enhance its read-across functionality. This integration will enable the software to retrieve data-rich substances from the EDT database, aiding in the safety evaluation of query (data-poor) substances. Additionally, features such as similarity scoring between the query compound and its analogs will be incorporated. We will carefully consider the peer reviewers' suggestions during the software development process.
   c) Promote the EDT through workshops, conferences, and social media and provide public training for the EDT.
   The FDA plans to promote the EDT at upcoming workshops, conferences, webinars, and on social media. We have already conducted training for FDA employees on the EDT and are considering offering public training sessions as well.
2. **Specific Clarifications and Updates:**
   a) Improve readability by using square brackets for additional context and placing sub-sub-questions on separate lines.
   In response to peer reviewers' comments on question 2, the FDA reformatted sub-

questions with many sub-sub-questions so that each sub-sub-question begins on a new line for improved readability. We also made additional edits to clarify all EDT questions to eliminate any ambiguity.

b) Address a question regarding the applicability of certain exemptions.
FDA notes that the exemption listed in EDT Q1c(i)) only applies to Q1c(i)).

c) Provide clarification for Q3f(iii).
At Q3f(iii)), FDA added "directly" to the question to clarify that the O^- must be directly bonded to N^+.

d) Address concerns regarding the coverage of certain toxic substances, particularly in Classes V and VI, and clarify the applicability of the EDT to nanomaterials and radioisotopes.
   i) One peer reviewer remarked, "It is not clear whether Classes V and VI are sufficiently protective for potent genotoxic carcinogens such as aflatoxins and nitrosamines or non-genotoxic Ah receptor agonists such as TCDD." The FDA believes that the Class V and VI TTCs are protective for all substances they encompass, including aflatoxins, nitrosamines, and TCDD. For instance, TCDD (CAS 1746-01-6), classified as a Class VI compound and known for its potency, produced a No Effect Level (NEL) of 0.001 µg/kg bw/day in a 2-year rat study (Kociba et al., 1978). Since the Class VI TTC of 0.00053 µg/kg bw/day is lower than TCDD's NEL, this demonstrates that the Class VI TTC provides adequate protection for TCDD and similar compounds. The FDA also notes that if future data indicate a need to update (e.g., lower) the Class V or VI TTC, it will make the necessary adjustments and communicate the scientific rationale behind the change.
   ii) As stated elsewhere in this document, nanomaterials and radioisotopes are not in the applicability domain of the EDT.

e) Discuss the inclusion of naturally occurring toxins, the overall comprehensiveness of the toxin space covered, and the placement of yessotoxins into Class VI.
One of the reviewers stated that they agreed with the highly potent brevetoxins and ciguatoxins being placed into Class VI, however, they believed that the structurally similar yessotoxins (referred to as "yessetoxins" by the reviewer) are much less potent and "Class VI would be overprotective". FDA notes that yessotoxins (YTXs) are closely related to ciguatoxins and despite dozens of YTXs having been identified, the oral toxicological potential of YTXs has not yet been completely clarified and, as the reviewer stated, their mode of toxic action is unknown (Paz et al., 2008). Only about 10% of YTXs have undergone somewhat 'useful' toxicological studies and no subchronic, chronic, or carcinogenicity studies are available. The longest duration oral study FDA could find was a 7-day study with yessotoxin (YTX, 2 mg/kg bw/day), homoYTX (1 mg/kg bw/day), and 45-hydroxy-homoYTX (1 mg/kg bw/day) in mice (Tubaro et al., 2004). While "only ultrastructural changes in the cardiac muscle cells near the capillaries, such as package of rounded mitochondria and alteration of the cells boundary were observed, without any increase of lactate dehydrogenase, an index of cardiac damage," it is FDA's opinion that these studies were too short in duration to confidently predict the chronic oral toxic potential of all YTXs. Moreover, according to more recent studies, "YTX can cause genotoxicity and induce mitotic catastrophe which can lead to different types of cell death"

(Korsnes and Korsnes, 2017, 2018). Considering the lack of subchronic and chronic/carcinogenicity studies in laboratory animals, the unknown MOA of YTXs, evidence of genotoxicity, and their close structural similarity to ciguatoxins, until more data and information become available on YTXs, they will be placed into Class VI to err on the side of caution.

The FDA acknowledges that the coverage of natural toxins is incomplete. While we aimed to include all toxins with available toxicological data, there remains a variety of natural toxins that we hope to better address in future updates as more in vivo and in vitro data become available.

f) Determine the best place to provide a definition for the term "connector". Connector is now defined in the definition section where all other definitions are provided.

g) Correct the typos listed, rephrase certain statements, and update some examples. Corrections included:
   i) Regarding whether "iv) If yes to e(i)) or e(ii)), assign to Class II." At EDT Q2 should read "iv) If yes to a) and e(i)) or e(ii)), assign to Class II.": the FDA agrees and has made the corresponding edit.
   ii) Regarding whether "v) If yes to a) but no to b), c), and d), assign to Class V." should read "v) If yes to a) but no to b), c), d), and e), assign to Class V.": the FDA agrees and made the corresponding edit.
   iii) Regarding whether "Q43c)" is a typo and should read "Q33c)" at 4.4.3.57 (now 4.4.3.58): the FDA agrees and corrected the typo.
   iv) Regarding whether "Q43a(i))/Q43a(iii))" is a typo and should read "Q34a(i))/Q34a(iii))" at 4.4.3.58 (now 4.4.3.59): the FDA agrees and corrected the typo.
   v) Regarding the suggestion to use the same statement, "but these cannot be a part of a heterocyclic ring itself," in Q3c(ii) as in Q3c(i) for consistency, the FDA agrees and has made the corresponding edit in the post-validation EDT.
   vi) FDA corrected punctation and a typo at Q3f(vi)).
   vii) FDA added missing closing parenthesis to Q3g(viii)) and Q3e(iii)).
   viii) Updated the example for "no" at the end of EDT Q5.
   ix) Regarding whether "Examples for Q3e(iii)" in the figure at Q6e(iii) should read "Examples for Q6e(iii)": the FDA agrees and corrected the typo.
   x) The red color used in the example structure drawings after Q18b) was change to green to avoid confusion as red in the post-validation EDT is used to mark changes compared to the pre-validation EDT. No red was used in any of the drawings to avoid confusion.
   xi) At Q28n(ii)), "(other than in the previous sub-sub-question)" was edited to "(other than those captured at Q28n(i))" for clarity.

References:

Paz, B., Daranas, A. H., Norte, M., Riobó, P., Franco, J. M., & Fernández, J. J. (2008). Yessotoxins, a group of marine polyether toxins: an overview. *Marine Drugs*, 6(2),

73-102.
Tubaro, A., Sosa, S., Altinier, G., Soranzo, M. R., Satake, M., Della Loggia, R., & Yasumoto, T. (2004). Short-term oral toxicity of homoyessotoxins, yessotoxin and okadaic acid in mice. *Toxicon*, 43(4), 439-445.
Korsnes, M. S., & Korsnes, R. (2017). Mitotic catastrophe in BC3H1 cells following yessotoxin exposure. *Frontiers in cell and developmental biology*, *5*, 30.
Korsnes, M. S., & Korsnes, R. (2018). Single-cell tracking of A549 lung cancer cells exposed to a marine toxin reveals correlations in pedigree tree profiles. *Frontiers in oncology*, 8, 260.
Kociba, R. J., Keyes, D. G., Beyer, J. E., Carreon, R. M., Wade, C. E., Dittenber, D. A., ... & Humiston, C. G. (1978). Results of a two-year chronic toxicity and oncogenicity study of 2,3,7,8-tetrachlorodibenzo-p-dioxin in rats. *Toxicology and applied pharmacology*, 46(2), 279-303.

One reviewer submitted a multi-page combined comment instead of individual feedback for the phase 2 questions. The FDA extracted relevant comments from this section and addressed them under the appropriate phase 2 questions. However, some comments from the combined response did not align with any phase 2 questions. The FDA will address these comments here:

Comment:

"For the phthalates, the evidence for lower potency to induce developmental effects in the shorter phthalates is perhaps not sufficiently strong to have them in Class III as compared to Class IV (e.g. DEP, a suspected ED). Sometimes, it is not clear why some closely related compounds are assigned to different classes while their points of departure would not support this. For instance, MEHP is Class III although its LEL is lower than that of its parent DEHP in Class IV."

FDA response:

The sub-sub-questions within Q34a) are designed to group phthalates based on similarities in structure, metabolism, mode of toxic action, and toxicity endpoints. MEHP (CAS 4376-20-9) is included in Q34a(ii) and assigned to Class III, along with nine other related substances in the combined EDT database. The FDA acknowledges that even among closely related compounds, there can be variations in toxic potential. Therefore, it is important not to isolate a single compound within any groups based on a seemingly low NEL compared to the NEL of other group members but to consider the NELs of all related substances within that group. MEHP has the lowest LEL, and consequently the lowest NEL, among the compounds included in Q34a(ii)). Given the context of all closely related compounds in this category/captured at Q34a(ii)), the Class III assignment appears appropriate.

The FDA notes that while phthalates are relatively well-studied compared to many other compound groups, there are still significant data gaps that need to be addressed. We hope

that more data on phthalates and related substances becomes available in the future, allowing us to refine the sub-sub-questions related to these compounds.

Comment:

"In Question 1i, the instructions are to disregard "the following commonly encountered and relatively nontoxic or of low toxicity i) metal counterions: sodium, potassium, calcium, magnesium, barium, aluminum, titanium, zinc, manganese, copper, iron, and bismuth". The relatively high EDT Class I TTC value may become problematic for counterions like aluminium, manganese and copper as their health-based guidance values could be exceeded. In addition, the formation of complexes with chemicals falling in Class I may lead to enhanced bioavailability of the metal counterion."

FDA response:

The FDA notes that elements such as aluminum, manganese, and copper are not within the applicability domain of the EDT when they are not present as counterions to an organic ion. In these cases, none of the EDT TTCs apply to these elements or their inorganic derivatives. They are only included in the applicability domain when they serve as counterions to an organic ion that is in the applicability domain of the EDT.

The TTC of the organic compound applies to the entire complex, including both the organic ion and the inorganic counterion, not separately. For example, in the case of copper phthalocyanine (CAS 147-14-8), an EDT Class I substance with a molecular weight of 576.08 g/mol, the Class I TTC of 385 µg/kg bw/day applies to copper phthalocyanine (as a whole), and not to copper and phthalocyanine separately. Given that the molecular weight of copper is 63.55 Da, copper contributes approximately 11% of the total weight of the compound. Thus, while the Class I TTC is 385 µg/kg bw/day for the entire compound, it effectively allows for only 42 µg/kg bw/day for copper (11% of the TTC value).

According to the National Institutes of Health (NIH, 2022), the tolerable upper intake level (UL) for copper in individuals aged 19 and older is 10,000 µg/day, or 167 µg/kg bw/day for a 60 kg person, which means the UL for copper is not exceeded. Moreover, according to the NIH website, some foods, considered safe, contain very high levels of copper (e.g., beef liver: 12,400 µg/serving and oysters: 4,850 µg/serving) and dietary supplements contain up to 15 mg (15,000 µg) per day of copper.

The most prevalent inorganic metal counterions are Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}. In our extensive EDT database of over 3,100 compounds, the lowest classification for a compound containing Al^{3+} counterion is Class III, while for Mn^{2+}, it is Class V—both of which have much lower TTCs than Class I. In our database, only two compounds with copper fall into Class I: C.I. Phthalocyanine Green (CAS 1328-53-6) and copper phthalocyanine (CAS 147-14-8), both of which are primarily used as colorants and have high NELs; way above the Class I TTC. All other copper-containing compounds fall into Class IV or V in the EDT database.

The FDA notes that when a health-based value or regulatory limit for a substance exists, it takes precedence over the safe levels predicted by the EDT. The EDT does not override existing limits, rules, or regulations.

In summary, we believe that the EDT TTCs are protective for the above listed metal counterions when they are a present as counterions to an organic ion within the applicability domain of the EDT. They are outside the applicability domain of the EDT when present as inorganic ions without an organic counterion.

Reference:

Office of Dietary Supplements. (2022). Copper: Health professional fact sheet. National Institutes of Health. https://ods.od.nih.gov/factsheets/Copper-HealthProfessional/

Comment:

"The chemical bisphenol S would be redirected from Q 36 to Q 41. No clear indication of further redirection is given and under Q 41, BPS would be assigned to Class III. In the EDT DB it is assigned to Class II."

FDA response:

Regarding bisphenol S (BPS, CAS 80-09-1), its path through the EDT is as follows: 1N, 2N, 3N, 4N, 6N, 7N, 9N, 10N, 23N, 29Y, 33N, 34N, 35bY, 36a,bY, 41N, 42N, 43N, 44N, 45N, 28N where N stands for a no response at the question and Y stands for a yes response. At Q41, based on the structural features of BPS, the answers to both sub-questions a) and b) are no. The instruction at the end of Q41 states, "ii) If no to a) and b), proceed to Q42." After answering no at Qs 42, 43, 44, and 45, the substance is directed to Q28, resulting in a final classification of Class II. Therefore, we are unclear about the peer reviewer's comment.

IV. APPENDIX A: DESCRIPTION OF SCHEMA 1

Schema 1 visually represents the decision-making flow within the EDT, outlining how responses to each of the 47 main questions guide the classification of a compound. For users of assistive technology or readers requiring a text-based version, the following description outlines the logic sequence step-by-step.

Detailed Description:

1. **Question 1**
   - Yes: Assign to Class I
   - No: Go to Q2
2. **Question 2**
   - Before assigning the substance to a class at Q2, the user is directed to crosscheck against Qs 5 and 6; that is, if due to the presence of a toxic element addressed at Q5 or a structural moiety of high toxic potential at Q6 the substance would go to a higher class at either Q5 or Q6 than it would at Q2, the substance will be assigned to the highest class it would get at either Q2, Q5, or Q6.
   - Yes: Assign to Class II, III, V, or VI.
   - No: Go to Q3
3. **Question 3**
   - Before assigning the substance to a class at Q3, the user is directed to crosscheck against Qs 5, 6, 43, and 44; that is, if due to the presence of a toxic element addressed at Q5 or a structural moiety of high toxic potential at Q6 the substance would go to a higher class at either Q5 or Q6 than it would at Q3, the substance will be assigned to the highest class it would get at either Q3, Q5, Q6, Q43, or Q44.
   - Yes: Assign to Class II, III, IV, or V.
   - No: Go to Q4
4. **Question 4**
   - Yes: Go to Q5
   - No: Go to Q6
5. **Question 5**
   - Yes: Go to Q6 or assign to Class II, III, IV, V, or VI
   - No: Assign to Class IV, V, or VI
6. **Question 6**
   - Yes: Assign to Class I, II, IV, V, or VI
   - No: Go to Q7
7. **Question 7**
   - Yes: Go to Q8 or assign to Class II, III, IV, or V
   - No: Go to Q9
8. **Question 8**
   - Yes: Assign to Class III, V, or VI

- No: Go to Q11 or Q33 or assign to Class IV

9. **Question 9**
   - Yes: Assign to Class I
   - No: Go to Q10
10. **Question 10**
    - Yes: Go to Q11
    - No: Go to Q23
11. **Question 11**
    - Yes: Go to Q1, Q12, Q13, Q14, or Q33
    - No: Go to Q12 or Q33
12. **Question 12**
    - Yes: Go to Q1, Q10, Q30, or Q33 or assign to Class IV
    - No: Go to Q13
13. **Question 13**
    - Yes: Go to Q14
    - No: Go to Q15
14. **Question 14**
    - Yes: Go to Q33 or assign to Class II, III, or V
    - No: Assign to Class IV
15. **Question 15**
    - Yes: Go to Q28
    - No: Go to Q16
16. **Question 16**
    - Yes: Assign to Class III
    - No: Go to Q17
17. **Question 17**
    - Yes: Go to Q19
    - No: Go to Q18
18. **Question 18**
    - Yes: Assign to Class III or V or go to Q28. If no at Q28, go to Q47 for final class assignment.
    - No: Go to Q28
19. **Question 19**
    - Yes: Assign to Class III, IV, or V
    - No: Go to Q20
20. **Question 20**
    - Yes: Go to Q21 or Q47 or assign to Class III, IV, or V. Before assigning the compound to a class at Q47, crosscheck against Q43. Assign the substance to the highest class it would get at either Q47 or Q43.
    - No: Go to Q21
21. **Question 21**
    - Yes: Go to Q28

- No: Go to Q22

22. **Question 22**
    - Yes: Assign to Class III
    - No: Go to Q47, but crosscheck against Q43. Assign the substance to the highest class it would get at either Q43 or Q47.
23. **Question 23**
    - Yes: Go to Q24
    - No: Go to Q29
24. **Question 24**
    - Yes: Go to Q1 or Q25 or assign to Class I or III
    - No: Go to Q25
25. **Question 25**
    - Yes: Assign to Class III
    - No: Go to Q26 or Q47
26. **Question 26**
    - Yes: Go to Q27
    - No: Go to Q47. Before assigning the substance to a class at Q47, crosscheck against Q28. Assign the substance to the highest class it would receive at either Q47 or Q28.
27. **Question 27**
    - Yes: Assign to Class IV or V
    - No: Go to Q28
28. **Question 28**
    - Yes: Go to Q1 or assign to Class III, IV, or V
    - No: Assign to Class II or III
29. **Question 29**
    - Yes: Go to Q33
    - No: Go to Q30
30. **Question 30**
    - Yes: Go to Q31 or assign to Class I, III, or IV
    - No: Go to Q47. Before assigning the substance to a class at Q47, crosscheck against Q28. Assign the substance to the highest class it would receive either at Q28 or Q47.
31. **Question 31**
    - Yes: Assign to Class III
    - No: Go to Q32
32. **Question 32**
    - Yes: Assign to Class III or IV
    - No: Go to Q28. Before assigning the substance to a class at Q28, crosscheck against Q24. Assign the substance to the highest class it would receive either at Q28 or Q24.
33. **Question 33**

- Yes: Assign to Class IV or V
- No: Go to Q34

34. **Question 34**
    - Yes: Go to Q1 or assign to Class III or IV
    - No: Go to Q35
35. **Question 35**
    - Yes: Go to Q36 or Q38
    - No: Go to Q47
36. **Question 36**
    - Yes: Go to Q37 or Q41
    - No: Go to Q47
37. **Question 37**
    - Yes: Assign to Class III or V
    - No: Go to Q47
38. **Question 38**
    - Yes: Assign to Class IV
    - No: Go to Q39
39. **Question 39**
    - Yes: Assign to Class III
    - No: Go to Q40
40. **Question 40**
    - Yes: Assign to Class I or III
    - No: Go to Q41
41. **Question 41**
    - Yes: Assign to Class III
    - No: Go to Q42
42. **Question 42**
    - Yes: Assign to Class II or III
    - No: Go to Q43
43. **Question 43**
    - Yes: Assign to Class III, IV, or V
    - No: Go to Q44
44. **Question 44**
    - Yes: Assign to Class III, IV, or V
    - No: Go to Q45
45. **Question 45**
    - Yes: Go to Q46
    - No: Go to Q28
46. **Question 46**
    - Yes: Assign to Class II or III
    - No: Go to Q47
47. **Question 47**

- Yes: Go to Q11, Q19, or Q35 or assign to Class I, II, III, or IV
- No: Assign to Class IV

V. APPENDIX B: INDIVIDUAL PEER REVIEWER COMMENTS

Questions for section 1 (The Expanded Decision Tree (EDT)):

Question 1: Has FDA clearly explained, with adequate examples, all guidelines and definitions for use with the EDT? If not, please provide suggestions for alternate text and/or additional examples.

REVIEWER	COMMENT
Reviewer #1	I am basing this response on my interpretation of Section 1.5 (please clarify if this is incorrect). FDA has done an excellent job in describing the chemistry basis of the EDT in many aspects. I found the description of chemistry in Section 1.5 to be clear and very logical. This is appropriate for use in a toxicological risk assessment tool such as the EDT. The description inevitably requires the reader to have some background in organic chemistry, without which the reader will not have the full understanding of the subtlety and sophistication of the scheme. I do not see that this is a problem, if the user of the scheme required detailed interpretation and they did not have that level of chemistry, then they would need to seek it from a more qualified person. Taking my own experience as an example, I can understand the chemistry, but I would not be able to comment on whether the chemistry is correct or appropriately set out (I have no reason to believe that it is not). To be critical of Section 1.5, it may be useful to include structures for classes A-F (Aliphatic – Aromatic ring). This may be especially helpful if and when the scheme is coded computationally. I also wonder whether this information may be easier to comprehend in a table, for instance with headings such as (although this may not be possible or practical): Class | Title | Description | Detail | Relating to EDT Question | | | | | | | | | | | | Section 1.5 contains a variety of different aspects of chemistry, going from fundamental classes of compounds such as being aliphatic or aromatic to different types of functional groups (see Section H) including very specific functional groups including reactive moieties etc. It might be more logical to organize this section into major structural features, different types of scaffolds (e.g., bridged compounds, zigzag etc.) and then organize the functional groups in a logical manner. Another aspect that I found lacking from the whole EDT is a section describing the applicability domain of the scheme (I comment on this elsewhere below). It may not be appropriate to evaluate this with regard to Section 1.5, but it is a key definition of the scheme. For instance, is there a molecular weight cut off? The term “guidelines” is used in Section 1.5, I do not believe there are real guidelines provided here but these are definitions. For the avoidance of doubt, it may be appropriate to remove the term “guidelines” or clearly denote what it

Question 1: Has FDA clearly explained, with adequate examples, all guidelines and definitions for use with the EDT? If not, please provide suggestions for alternate text and/or additional examples.	
REVIEWER	**COMMENT**
	means. Overall, this Section 1.5 is very comprehensive which could be made clearer with organization of the information into a table and different levels of structural definition. It would also benefit from a statement at the beginning of the section that defines clearly what is the purpose of this section and how it is set out, with the expectations but the reader would gain from it - for instance it could be stated that there is a requirement for a knowledge of organic chemistry to interpret information. What we do not want is put off non-chemists from using the EDT as they may feel it is a purely chemistry-based tool with no basis in toxicology (this is clearly not the case!!). Section 1.5, "I. Reactive moiety" It would be helpful to define what is meant by reactive here e.g., electrophilic, nucleophilic etc.
Reviewer #2	Overall, the guidelines and definitions for use with the EDT are clearly explained. However, it is noted that some definitions are composed specifically for the EDT questions and therefore do not have the same meaning as in the general literature, which created some challenges: • 1.5.J: EDT evaluates the organic salts and metal ion salts as their neutral forms. While this simplifies the decision process and may not impact the final classification, it contradicts the general understanding that valence states often determine the reactivity and therefore toxicity outcome. • 1.5.K. EDT defines "hydrolysis" as addition of element(s) of water to a molecule leading to either a different molecule or multiple molecules. EDT does not define "reduction" but refers to hydrolysis or reduction of functional groups in Figure 1. It would be useful to define both terms to improve illustration in Figure 1. • 1.5.N. "Corresponding" hydrolysis products are described and illustrated for primary and secondary alcohols. The structures have R1 to R5 substitutions but only R3, R4 and R5 are described. • 1.5.R. The text refers Bridgehead atoms to definition AA which may be a typo. • 1.5.X. The structures illustrate PHAs with the solo, duo, trio and quartet bonds which form bay and/or fjord regions. Please describe the difference between the bay and fjord regions.

Reviewer #3	For the most part all guidelines and definitions are clearly explained with adequate examples. Below are the areas where I think there could be some updates to make things a little clearer.
	• Section 1.5
	  ○ I think it might be best to rearrange the first 6 bullets to be ordered like this: Aliphatic, Acyclic, Alicyclic, Aromatic, Heterocyclic, Heteroaromatic.
	  ○ Alicyclic – maybe change to say: "means the presence of at least one ring composed of only carbon atoms with our without the presence of ring alkene(s) (i.e., C=C) that do not form an aromatic ring."
	  ○ Heterocyclic – maybe change to say: "means the presence of a ring with at least one ring atom that is not carbon…"

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REVIEWER	**COMMENT**
	○ Heteroaromatic – maybe change to say "means that the substance contains at least one ring, which is composed of at least one ring heteroatom…" ○ Pseudo-aromatic – maybe rearrange to say "…by incorporating the election pair of a functional group into an enolic double bond…" ○ Oxygenated functional group – maybe it'd be good to reference Figure 1 for users to refer to to see example structures. ○ Corresponding – should "Corresponding" be in bold? It's a little confusing that the word being defined is being used in the definition, maybe you could use "equivalent" instead? ○ Related – should "Related" be in bold? ○ Bridged – this is very minor, but it might be helpful to show the examples in the order they are defined (i.e., Bridged, Fused, Spiro, Singly bonded). ○ Electron pair donors – it might be useful to have a figure illustrating some of the SMARTs patterns outlined (e.g., ester, carboxylate, etc.) ○ Solo, duo, trio, quartet – I'm unsure what this definition is trying to say. Is it that each of the carbons must be bonded to an atom other than an atom in the aromatic ring, rather than "can be"? That is, the carbons have to be on the outside edge of the structure. Should there also be a definition of what the Bay and Fjord regions are?
Reviewer #4	A fundamental revision of the TTC databases and threshold definitions is very welcomed. To introduce the new concept of EDT, a very extensive and complete document was presented. • All definitions relevant to the chemistry contained in the different questions for the EDT are clearly and concisely presented. • The definitions (and guidelines) for the toxicological endpoints are less clear. In part this is due to the nature, but a clear position would have been helpful. For example, the concepts of NOAEL and NEL can be quite different. When was adversity taken into account and when where effects not necessarily linked to adversity considered? There is only reference to small increases in relative liver weight from metabolic loading, and this was excluded. I agree that the earlier literature used by Munro for their TTC was not always very clear on this point but still now there are discussions on when an effect is considered adverse and when it is considered adaptive. Perhaps the text could be clarified on this point, where an effect is judged as adverse. • Another point where the guidelines are not clear for the pre-validated EDT is on how the doses were calculated. The creation of the External Validation Database goes into much detail when administration of the chemical was not every day or when the compound had a lower purity. However, it is not stated whether the same approach was used to create the pre-validated EDT. More details here would be important. • A similar comment can be made regarding the chemicals used to pre-validated EDT. The issue of inaccurate and multiple CAS numbers, incomplete description of the chemical, etc. is described in the External Validation Database but not for the pre-validated EDT.

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REVIEWER	**COMMENT**
	• The examples provided give a good illustration of the information sought after in the questions. • A general comment on the presentation of the first document is that the individual questions could be better highlighted. It is easy to get lost in the document as the questions are only indicated by a number, and navigation because of the need to refer to other questions throughout the text is not easy. Would a flowchart be possible?

Question 2: Are all EDT questions clear as to which structural features they are describing? If no, please identify the question by its number, explain why you find the question ambiguous or confusing, and suggest alternative text to ensure that it is clear what kind of structural features the question is aiming to capture.	
REVIEWER	**COMMENT**
Reviewer #1	I found the EDT questions to be clear in terms of the structural features they intend to describe. What is described are structural alerts in their most simplistic form, in other words the molecular environment is not defined for these alerts, i.e., no substituents are defined. In the documentation for the EDT, it may be worth explaining this along with the rationale for doing so. There are advantages to this approach, namely that it is fundamental, and all identify any compound with a structural feature, there are also disadvantages as a structural feature may be buried within a molecule and may not be relevant for activity. The FDA appears to wish to maintain the ethos of the original Cramer decision tree (Cramer et al, 1978, *Food and Chemical Toxicology* 16: 255-276) which was based on chemical classes and groupings alone. This was quite revolutionary in so doing at that time, but there would be no problem in making the definition of chemical classes within the questions more sophisticated at the current time (I will make a comment related to this later). With regard to a previous comment made in Question 1, in the application of the EDT, it may be worth having a general statement or guidance on what the applicability domain of this is in terms of physio-chemical properties such as molecular weight. It should also be noted that the EDT is for single chemical structures. Clear guidance should be given before entering the EDT on how to address issues such as salts, counterions etc., i.e., should be neutralized form of a molecule be utilized? It would be helpful for each “question” to have a descriptive title to give the reader some insight into what it relates to.
Reviewer #2	Almost all the EDT questions are clear on the structural features and those with definitions are bolded for ease of reference. Colors are sometimes used to call out the structural features, and this is particularly helpful. However, Question 2 is extremely difficult to follow. In this question, the “=” can be a double bond or may mean “equal to”.
Reviewer #3	I think for the most part, the EDT questions are relatively clear which structural

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REVIEWER	**COMMENT**
	features they are describing. Below are some areas where I have some suggestions that may help to make things clearer: • Throughout – it'd be useful to have the sub-sub-bullets [e.g., i), ii), etc.] on a new line [like is done for the exceptions in Q1a)]. I think this would help a user more easily identify the different types of structures discussed in the question. • Throughout – double check that all terms defined in Section 1.5 are bold [e.g., "corresponding" in Q1a), Q1b), Q1c), etc.; "α" in Q1g), Q1h), Q3a) etc.]. • Throughout – I think it'd be useful to have a sub-heading saying under each question identifying where the assignment answers are [e.g., If yes assign to Class X, if no proceed to QX]. • Throughout – When examples in Figures contain substructures and/or functional groups present in the question, I think it'd be helpful to highlight the matching substructure. • In Q1 questions and Q3f) -, it'd be useful to underline the "and"/"or" before the sub-sub-bullets indicators [e.g., ii)]. This is used in Q2 and makes it easier to discern where the break is. • Q1e) – Should these be "A monosaccharide, or hydrolysable oligosaccharide or polysaccharide…" The way I read the current version seems to suggest the substance needs to contain all substituents to be considered a "Yes". • Q3g)ii) – azide – should the SMARTS pattern here be "-N^--N^+≡N"? • Q4 – for the albuterol sulfate example, I think it'd be helpful to include an illustration of the sulphate group with a note that it is to be disregarded. • Q6 b)ii) – even though there is no "i)" sub-bullet in Q6, it may be more easily understood if this was rewritten as "the compound does not have any of the skeletal structures listed in b)i)" so it's not confused for i). • Q6 c) – Is it the additional ring system that is to have the ≥2 oxygenated functional groups or the macrocyclic ring? • Q7 g)iv) – Should this be "a halogen"? • Q8 d) – Should the last line read "…ortho, meta, and para positions each (does not have to be on the same ring), and each ring must be substituted…"? • Q12 e) I would recommend switching the "4≤" to "≤4". • Q18 b) – Can any of the skeletons mentioned (up to the aromatic ring) be with or without the moieties mentioned (i.e., from the primary alcohols) or only the aromatic ring? This needs to be made clearer • Q18 assignments – ii) is missing "If yes" • Q22 – The wording of this is a little confusing, but I'm not sure how to reword it.

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REVIEWER	**COMMENT**
	• Q30 a) – It's not too clear what is being described here. Is this question saying the aliphatic chain can have ≥1 of the functional groups or only the alicyclic ring(s)? • Q45 – Can the substituents mentioned after alcohol be attached to the aromatic substituent mentioned just before (e.g., alicyclic ring, methylenedioxy, etc.), directly to the aromatic ring, or either? • Q47 f) – Final sentence, reword to "In addition, other than the tetrahydropyran ring that is fully substituted, all rings should have…"
Reviewer #4	The EDT questions are a mix of: • purely chemically defined questions (general structural features, elements contained in the structure, functional groups, etc.) (e.g., Q1,3-5, 12-33) • Structures associated families of known toxicants (e.g., Q2 for OP compounds, Q34 for phthalates) • Known biochemicals (e.g. Q1i) • Pharmacologically (toxicologically) active compounds of natural or man-made origin (e.g., Q6) From what I can judge, the purely chemically based questions are quite complete, whereas the questions dealing with naturally occurring compounds are limited to a few groups of pharmacologically (toxicologically) active compounds. This is also acknowledged in the document (p100). Will the incompleteness be compensated by the purely chemically based questions? The concern is that the most toxic compounds known are naturally occurring ones rather than man-made. It is difficult to reconstruct how the different questions are interlinked. Therefore, the fact that two different questions can address the same chemical and come to different class assignments may or may not occur when using the EDT. For example, TCDD can be answered in Q8(b) (assigned to Class VI) but also in Q18(a) (assigned to Class V). I tried to identify bisphenol through the questions. Q36, takes you to 41 then 42, then 43, then???

Question 3: Most questions place compounds into one of six classes of toxic potential depending on their structural features. Does the EDT place the type of compounds that are captured at each question into the appropriate class of toxic potential? If not, please explain why and provide a recommendation for the appropriate class of toxic potential.	
REVIEWER	**COMMENT**
Reviewer #1	I will use my response to this charge question to make some general comments that will hopefully address this question but also go on into other issues with regard to the EDT. The FDA may wish to accept or ignore these comments! It seems a simple question to ask if the EDT places compounds into the appropriate class of toxic potential. This is actually a very difficult question to

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REVIEWER	**COMMENT**
	tease apart and answer using the information given. To answer this question FDA needs to provide clear and upfront description of the six classes and their justification. The classes are defined in Tables 2 and 3 in Section 3.4. It almost seems as if the definition of classes becomes a self-fulfilling prophecy, were the classes defined *a priori*, or on the basis of the data? It is my understanding of the Cramer scheme that it did not set out to develop the classes on anything more than fundamental toxicological principles, less so on chemical groupings and data (although there is, of course, evidence of structure-activity in Cramer et al (1978). Whilst Fig 2 in Cramer et al (1978) plots some NOAELs, it was only Munro et al (1996, *Food and Chemical Toxicology* 34: 829-867) who started to put any significant numbers on the Cramer classes. So, I can agree that the EDT sets out six classes that go from “less toxic” (Class 1) to substances of very high toxicity (Class 6). My interpretation of the scheme, as described is that compound classes are generally in the right place, i.e. less toxic compounds are class 1, most toxic class 5 and 6. My feeling is that FDA has done a very good job in allocating chemicals classes to appropriate EDT classes. There were none that I felt uneasy with, although whether they are 100% correct will probably come with experience of using the scheme. I have no doubt that some refinements will be required at some point in the future, but what is describing appears to be an excellent starting point. To be honest, I found the technical description of the chemistry Section 1.7 really difficult to take in and comprehend. Whilst this is a criticism, it does not belittle or criticize the incredible job that has been done by FDA in organizing this. In terms of this review, this may come to life more in Phase II. With regard to presentation of the questions, I found myself going back and forth in the questions to determine which chemistries were associated with a particular EDT class. To help get the chemistry and EDT class across, possibly putting this information in a table, with chemistry linked to a class would be helpful and clearer. To fully understand the six EDT classes, it would be helpful to have a direct comparison with the Cramer scheme, i.e. is the chemistry in Cramer class 1 analogous to EDT class 1 etc. There is no reason Cramer and EDT classes should be the same, but it would help me understand the new classes, and I would imagine it would be helpful to others. Some other points: • FDA need to be very clear what this scheme is to be used for. There are a growing number of TTC schemes (and variations thereof) e.g. for skin sensitization, inhalation toxicity, ecological effects. I assume this scheme and the associated six classes relate solely to repeated, chronic oral exposure. • It would be helpful to define whether (assuming they do) the six classes replace the requirement to deal with DNA reactive compounds and the Cohort of Concern separately. • There is no easy way to demonstrate and document such a complex and detailed chemical analysis – perhaps this should be summarized in a

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REVIEWER	**COMMENT**
	publication with full access to the information via a computational / software application. I am thinking of perhaps of how meta information / data are held for structural alerts e.g. in Derek Nexus or the OECD QSAR Toolbox. • The allocation of classes in the questions should be stated along with the description of chemistry, as it is, for some questions I was required to go several pages forward to find the result. Also (this should be very easy for the software as noted above), it would be helpful to have the explanations provided in Appendix 1 with the questions – I ended up flicking back and forward to find / interpret this information. • Ultimately it would be great to have mechanistic information linked to the questions, even relevant AOPs (accepting there will be gaps in AOP coverage). There has long been appreciation of the potential value of a mechanism / mode-based classification system – although I am not sure how possible that is in practical terms. A final comment here is rather fundamental. The EDT is by its name a "decision tree", however no tree is provided. I am thinking here how familiar we are with the CDT, in particular Figure 1 in Cramer et al (1978), I was expecting to see something similar in this report. This actually raises the question of whether a yes / no decision is appropriate. What happens if a molecule contains two "alerts", the first is less toxic and the molecule is assigned to that class early in the decision tree? I fully understand the strength of the decision tree approach, but now we can do things differently – why not assess the molecule against all alerts and use the most conservative classification? In this way, the classification is most protective, and it would be transparent. I realize we are very familiar with the decision tree approach, but it does not mean that it should applied in the new scheme.
Reviewer #2	Section 1.8 provides the rationale for the EDT classification based on structural features and predictions for metabolic activation/detoxification/biological reactivity. The 47 questions in the pre-validation EDT illustrate how appropriate EDT Classes are determined. Appendix 1 provides the short explanation for each of the 47 question and sub-questions. Without Appendix 1, rationale behind each question can be easily lost. Section 1.8 also defines Classes I through VI with high level examples for each class. The significant toxicity endpoints are addressed in the Charge Questions, with the exception of skin sensitization and systemic hypersensitivity. If no consensus is reached whether or not EDT should address these endpoints, it would be good to highlight these for future research efforts. While most questions are challenging for a non-chemist, Question 28 is particularly difficult. This question is a terminal question that covers a large number of biologically reactive moieties with increased potential for toxicity and assigns them to Classes III, IV or V. Appendix 1 gives a detailed description of the compounds and the associated mechanisms of action and toxicity endpoints (e.g. cardiovascular toxicity, neurotoxicity, carcinogenicity, oxidative stress). It

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REVIEWER	**COMMENT**
	would be helpful to provide the basis for sub-questions i), ii), iii), iv) and v) in ranking these toxicity endpoints into Classes III, IV or V.
Reviewer #3	I think this question is probably best answered after I have received Appendix 2. That way I can look at some of the chemicals and go through the questions to assign a subset of the chemicals and compare what I get to the assignments in the Appendix.
Reviewer #4	This question is difficult to answer. The document does not provide many examples with clear class assignments. Where it is the case, classification falls in line with their previous one under the extended Cramer one. Particularly helpful would have been a more complete list, and perhaps more importantly the chemicals falling outside the 95th percentile. There is some unclarity here regarding some endogenous phosphorylated compounds that are formed from precursor macromolecules (phospholipids) or further phosphorylated in the process. Some are captured by Q1i, others by Q2c. Examples: IP3, IP4, IP6. In the former case, they are classified as I. In the latter case, this leads to class III, and this an overclassification. An example would be IP6 (phytic acid). Q18 puts TCDD and congeners into class V which will not be sufficiently protective (0.031 ug/kg bw/day vs 0.25 pg/kg bw/day). Subsequent check with the EDT spreadsheet has TCDD listed with a NEL of 1 pg/kg bw.

Question 4: Commonly, structurally related compounds (e.g., γ-diketones) can have common toxicological endpoint (in this case γ-diketone type neurotoxicity). Compounds that can either hydrolyze and/or metabolize to these compounds can exhibit the same type of toxicity. FDA aimed to capture hydrolytic and metabolic precursors of structurally related compounds with similar toxicities at numerous questions. Are there any questions where you can suggest any possible metabolic and/or hydrolytic precursors to the types of compounds addressed questions that are currently not mentioned/captured in the question?	
REVIEWER	**COMMENT**
Reviewer #1	The FDA should be congratulated on the completeness of the coverage of the EDT, especially capturing hydrolytic and metabolic precursors. This is not a particular area of personal expertise for myself, so I am unable to comment other than to say there is nothing obvious to me that has been omitted.
Reviewer #2	The proximate carcinogens may help with this question. Perhaps they can be captured in the questions with PAHs.
Reviewer #3	There are no questions where I can suggest other metabolic and/or hydrolytic precursors that aren't currently captured.
Reviewer #4	The EDT does a very good job in assigning appropriate classes to reactive intermediates. The above mentioned 2,5-hexanedione example is assigned to

Question 4: Commonly, structurally related compounds (e.g., γ-diketones) can have common toxicological endpoint (in this case γ-diketone type neurotoxicity). Compounds that can either hydrolyze and/or metabolize to these compounds can exhibit the same type of toxicity. FDA aimed to capture hydrolytic and metabolic precursors of structurally related compounds with similar toxicities at numerous questions. Are there any questions where you can suggest any possible metabolic and/or hydrolytic precursors to the types of compounds addressed questions that are currently not mentioned/captured in the question?	
REVIEWER	**COMMENT**
	class IV. Both Toxtree and the QSAR Toolbox also assign it to Cramer class III. Here the EDT shows its strength as it picks up the precursor and correctly assigns it to class IV whereas both Toxtree and QSAR Toolbox put it into Cramer Class I. The intermediate metabolite 2-hexanol is put in Cramer class II and I, respectively. EDT assigns it Class III (Q24 – if I am correct). For the further metabolite, 2-hexanone, the QSAR toolbox assigns it to Class I, Toxtree to Class II, and EDT to Class IV. The related 1,2-diethylbenzene that manifests the same type of neurotoxicity is in Cramer Class I but EDT Class IV. The neurotoxicity of hexane is well documented. My understanding is that long term exposure to high concentrations is necessary to elicit human toxicity. Would a Cramer Class I still be protective. The US National Institute for Occupational Safety and Health (NIOSH) has set a recommended exposure limit (REL) for n-hexane of 50 ppm (180 mg/m^3 over an 8-hour workday. Cramer Class I would be 2 orders of magnitude lower (let's ignore routes of exposure for the moment). Therefore, is the need to assign all hexanes to EDT class IV justified? The problem often encountered in practice is that exposure will not be directly to the reactive intermediate but to appropriate precursor molecules. The reactive molecules described in the different questions may be too unstable to be of relevance from an external exposure point of view. This issue of metabolism is addressed for some families of compounds (e.g. epoxide formation from PAHs) where the pathway is well-established but may miss many other parent compounds. Would there be room to consider linking the EDT questions to third-party predictors of metabolism? Prediction of metabolism would facilitate the inclusion of a number of substances in the questions dealing with reactive chemicals if it could be predicted that they would be formed as part of their metabolism.

Question 5: Are the example structures provided after each EDT question correct and adequate for understanding what type of compounds the question aims to capture? Are there different or additional example structures for any of the questions that would help increase the understandability of the question?	
REVIEWER	**COMMENT**
Reviewer #1	I am very grateful to the FDA for including chemical structures alongside the questions. This is a great help to me (as chemically aware scientist, but not a chemist). I am sure it will be helpful to many others. I believe the compounds

Question 5: Are the example structures provided after each EDT question correct and adequate for understanding what type of compounds the question aims to capture? Are there different or additional example structures for any of the questions that would help increase the understandability of the question?	
REVIEWER	**COMMENT**
	provided are correct (I did not see anything incorrect). It may be helpful to have chemical / compound classes (which may help define domains) and individual exemplar compounds (most / all of which would never be dealt with by TTC as they will have data). Again, the chemical classes could be linked to mode / mechanism, but also maybe to HPVC classes and other means of classification and inventories.
Reviewer #2	The example structures are challenging for non-chemists. In compounds with substitutions, it would be helpful to label the carbon positions. An example is the structure in Question 32 d), 7-oxocyclohepa-1,3-dien-1-yl propionate.
Reviewer #3	The example structures appear to be correct and adequate for understanding the types of compounds covered by the questions. • As mentioned above for Question 2 – I think something that could help increase the understandability of the question, especially where several substructures are listed [e.g., Q2b)], would be to highlight the substructure in the example chemical. I think this would help to identify the substructures much more easily. • Q12 e) - In the example here, I think it would be useful to also use a substance with an ellagic acid backbone as an example, because it was mentioned in Q12 a) that these substances are dealt with here. • Q47 g) – Should the example structures here be in Kekule form to match the other aromatic rings?
Reviewer #4	Generally, yes but examples for where the answer to the question is negative can be difficult to reconstruct as it can lead to multiple questions with negative answers (e.g. dapsone).

Question 6: Are there any congeneric groups that the EDT does not adequately address, but for which enough safety data exist that could serve as the basis of additional EDT questions to address these groups? If yes, please identify and provide all toxicological data for the congeneric group(s) that may form the basis of one or more additional questions. If possible, please propose the wording for such additional EDT questions. (Substances within a congeneric group are structurally and metabolically similar.)	
REVIEWER	**COMMENT**
Reviewer #1	I do not believe that there are any obvious congeneric groups with data that have been omitted from the EDT. A couple of thoughts: • If not attempted (it may be in Phase II), it would be a useful exercise to map the EDT questions to available data e.g. take the compounds in a database and determine which question and EDT class they relate to.

Question 6: Are there any congeneric groups that the EDT does not adequately address, but for which enough safety data exist that could serve as the basis of additional EDT questions to address these groups? If yes, please identify and provide all toxicological data for the congeneric group(s) that may form the basis of one or more additional questions. If possible, please propose the wording for such additional EDT questions. (Substances within a congeneric group are structurally and metabolically similar.)

REVIEWER	COMMENT

	• Mapping the EDT classes will probably help with the definition of applicability domain e.g. which types of chemistry the EDT could be applied to.
	• Experience shows that many *in silico* models are used well beyond their intended domain and purpose. Therefore, should the EDT aim to help with e.g.
	  ○ Sugars, amino acids – especially those considered GRAS
	  ○ Polymer components
	  ○ Botanicals – expand the knowledge to more botanical classes
	  ○ Pharmaceuticals – there are many data and knowledge of mechanisms etc.
	  ○ Nanomaterials
	  ○ Biocides – consideration of pesticide data and mechanisms
	  ○ Natural toxins e.g. mycocystins
	The final two examples (biocides / natural toxins) may be particularly relevant to food.
	The need for animal data to extend the EDT further will be restrictive, thus there may be good reason to extend on a mechanistic basis, e.g., using omics, NAM etc. data.

Reviewer #2	Siloxanes are often detected as extractables/leachables from food packaging materials. There are linear and cyclic structures with a wide molecular weight range. There is a wealth of toxicology information that can be leveraged for EDT questions.
Reviewer #3	I'm not sure whether there are any other congeneric groups that have enough safety data where a question could be written that isn't covered by the EDT.
Reviewer #4	For metals and organometals, additional questions on their solubility and pKa values under physiological conditions to predict their ADME properties would allow to refine the grouping. This would allow to address the considerable differences in the toxicity of some metal salts, e.g. Ba salts.

Question 7: Should any questions be further subdivided to ensure a more refined grouping of related substances? If yes, please suggest wording for the refined question(s) and provide the data justifying the suggestion.

REVIEWER	COMMENT
Reviewer #1	The FDA has done an incredible job in defining and dividing the questions, resulting in very fine granularity. This shows very great expertise and thought.

Question 7: Should any questions be further subdivided to ensure a more refined grouping of related substances? If yes, please suggest wording for the refined question(s) and provide the data justifying the suggestion.	
REVIEWER	**COMMENT**
	There were no refinements to the classes that seemed obvious to me at this time.
Reviewer #2	The groupings appear adequate at this point. When the EDT database is shared in Phase II, we can revisit this question.
Reviewer #3	I'm not sure. This may be something I can better answer after looking at the chemicals in Appendix 2.
Reviewer #4	See answer for Q6.

Question 8: Are there any terms used in the EDT questions that should be added to the guidelines and definitions section to help users of the EDT? If yes, what additional terms should we define?	
REVIEWER	**COMMENT**
Reviewer #1	The guidelines and definition focus on chemistry and defining that. The chemistry does not need any more definition in my opinion. Here I summarize some thoughts expressed above, as well as others: • The guidelines could give an overall statement of the use of the EDT and the information it can provided i.e. TTC for oral repeat dose / chronic exposure etc. • An applicability domain for the EDT could be given. • I would have preferred to see a description of the six EDT classes – or at least an overview / brief explanation – before reading the questions. Information is given in Section 1.8 and I only read that after reading the questions, so I was trying to interpret the questions without knowing what the EDT classes meant! I realize that answering this question is really a moot point. Most users will simply go to a usable piece of software and never consider the original description in the paper. Other comments that do not fit elsewhere: • Sections 1.1 and 1.2. The start of these sections would benefit from shorter sentences, more punctuation etc. • Section 1.2, paragraph 3. It could be stated that there have been updates of the ToxTree implementation of CDT, as well as other implementations e.g. VEGA, COSMOS NG, OECD QSAR Toolbox etc.
Reviewer #2	Section 1.5 provides the guidelines and definitions which are very helpful for the EDT questions. Many endogenous and exogenous compounds may exist as stereoisomers and enantiomers, e.g flavonoids in Question 15. It may be helpful to add definitions for the common isomeric forms.
Reviewer #3	Maybe it would be useful to define the terms: • Conjugated. • Dimer • Organyl

Question 8: Are there any terms used in the EDT questions that should be added to the guidelines and definitions section to help users of the EDT? If yes, what additional terms should we define?	
REVIEWER	**COMMENT**
	• Connector
Reviewer #4	The explanations to the different questions given under Appendix 1 is very useful but should be incorporated into the questions themselves. Under Appendix 1 a number of explanatory terms such as receptors, pharmacological activity are used. As for the bullet point above, it would be good to have these concepts included in the questions themselves as they represent the rationale for several of the questions.

Questions for section 2 (The Expanded Decision Tree Chemistry, Toxicity, and Metabolism Database (EDT DB)):

Question 9: Has FDA clearly explained where the toxicological data found in the EDT DB were collected from? If not, what additional information should we provide?	
REVIEWER	**COMMENT**
Reviewer #1	FDA should be congratulated for the data collection exercise and attempting to record the data as clearly as possible. It is vital that all data are transparently collected. It is well known that this was a significant problem in the original Munro data set i.e. the source of data, especially the selection of the NO(A)EL was sometimes not clear. I strongly encourage FDA to include a transparent and fully documented and curated database of compounds, experimental details and the source of data (as, for instance, with COSMOS DB, see Yang et al (2017, https://doi.org/10.1016/j.fct.2017.08.043; 2021, https://doi.org/10.1016/j.comtox.2021.100175). This could even be linked to the questions / decisions i.e. which data relate to which alert etc. There is reference to the "EDT DB" at the end of Section 2.1, paragraph 3, and this must be made available and future-proofed, i.e. adequately described such that it could be updated by other workers not involved in this project.
Reviewer #2	Section 2.2 provides criteria for data collection. However, the source of the toxicological data is not explained until Section 4.2 Creation of the External Validation Database.
Reviewer #3	• Section 2.1: o When discussing searching the literature, please mention what search terms were used and what was used to search (i.e., PubMed, Google). For example, what types of information/studies were searched for, just subchronic and chronic? o Where was information on food contact substances gathered from? o When mentioning study details included, did the substance need to have all this information to be included in the EDT DB?
Reviewer #4	The description of the sources of data used for the pre-validated EDT. Some additional information would have been useful such as number of chemicals from the different sources, curation to get unique substances would have been

Question 9: Has FDA clearly explained where the toxicological data found in the EDT DB were collected from? If not, what additional information should we provide?	
REVIEWER	**COMMENT**
	useful. A table would help. The issue of inaccurate and multiple CAS numbers, incomplete description of the chemical, etc. is described in the External Validation Database but not for the pre-validated EDT. Missing is also the extensive discussion found in the External Validation Database but not for the pre-validated EDT on the approach used when administration of the chemical was not every day or when the compound had a lower purity. Limited information on inclusion and exclusion criteria is provided. The TTC approach comes with exclusion criteria regarding some groups of chemicals where the chemical space represented by the substances in the database was considered outside the domain of applicability. Examples are organosilicon substances, substances with a potential for bioaccumulation, endocrine disrupting chemicals, inorganic substances, nanomaterials, etc. Some of these groups have been incorporated in the EDT which is seen as positive but some more information on the underlying criteria would be helpful. This would also be very useful for those compounds that interact with nuclear receptors linked to endocrine activity. Some are addressed from a structural point of view in Q6 and Q34 (and Q33 for AhR ligands). While the TTC (Cramer class III) is found protective for the majority of man-made EDs, there are a number of exceptions. For example, among pesticides recently shown to display ED activity, the TTC would not be protective for chlorpyrifos (CAS # 2921-88-2), clodinafop (CAS #105512-06-9), flutamide (CAS # 13311-84-7).

Question 10: Has FDA clearly explained the study selection criteria and provided adequate information and/or data to support its opinion that these criteria are appropriate for data inclusion in the DB? If not, what additional information should we provide?	
REVIEWER	**COMMENT**
Reviewer #1	It is essential that the study selection criteria are reported and explained. This appears to be done and must be recorded in the database. I am not aware of any other information that should be provided as long as it is clear how a decision on e.g. the selection of a NO(A)EL, LO(A)EL etc. has been made. This should be more obvious when the data are recorded in the database, and I can attempt to follow how a decision has been made. The study selection criteria would be clearer if the list of criteria, e.g., in Section 2 were put in a table, this could include how many data were taken from each source, the source etc. Whilst it may appear trivial to FDA, something that could be reported is a “worked example” of how the value has been determined and the decisions made. It should be remembered that most TTC users do not appreciate the analysis of the original data. This could also allow for the easier updating of information if more data / knowledge become available and the criteria are updated.

Question 10: Has FDA clearly explained the study selection criteria and provided adequate information and/or data to support its opinion that these criteria are appropriate for data inclusion in the DB? If not, what additional information should we provide?	
REVIEWER	**COMMENT**
Reviewer #2	Section 2.2 explains the criteria for study selection in the original EDT database. Table 1 shows the distribution of studies in the original EDT DB based on the exposure route. While there are few subcutaneous, intravenous, and intraperitoneal studies individually, please consider combining these routes and analyze them collectively for parenteral route. Section 4.3.5 gives a brief description of the selection criteria for the studies in the external validation database. It is not surprising that there are conflicting data that require best judgement by the EDT panel of experts. The example provided for Tolclofos-methyl is very helpful. It would be good to understand the EDT panel peer review process.

Reviewer #3	• Section 2.2:
	  ○ I think this section would be easier to follow if there were headings relating to each criterion and they were discussed in turn, e.g., what species were allowed? What durations?
	  ○ If an NEL wasn't identified by a study, did you convert the LEL to an NEL?
	  ▪ This is mentioned in Section 3.3 (i.e., no LELs were converted to NELs), but it would be good to mention that here too
	  ○ In Section 4.3.6 it says that NELs in the EDT DB were adjusted based on the purity of the substance and dosing schedule, but that's not mentioned in this Section, please add that.
	  ○ What species were allowed for inclusion in the EDT DB?
	  ○ Page 80. line 22 – What number of animals are considered adequate to ensure statistical significance?
	  ▪ Did you go back and re-evaluate the statistical significance?
	  ○ Page 80, line 24 – Did you re-evaluate if the reported effects were adverse or not?
	  ○ Page 80, line 27 – "Limited reporting" on what? The number of animals, whether the effects were adverse, or something else? Please expand.
	  ○ Page 80, line 33 – is the NEL for the shorter duration study the original NEL or the adjusted NEL?
	  ○ Page 80, line 35-39 – What was the shortest duration allowed in the EDT DB? (Shortest in Munro was subchronic)
	  ○ Page 81, line 32-36 – This example is a little confusing as written, suggest to swap around as:
	  ▪ For example, for aliphatic, alicyclic, or aromatic ketones or hydrocarbons of sufficient molecular weight and lipophilicity that cause α_{2u}-globulin-type nephropathy, an endpoint not relevant to humans, and observed exclusively in male rats, we used the toxicological data (e.g., NEL and LEL values) for female rats only for inclusion in the EDT DB.
	  ○ Page 81, line 47-Page 82, line 4 – Both can't be true, either the offspring NEL was chosen if it was equal to the parent or the parent NEL was chosen.

Question 10: Has FDA clearly explained the study selection criteria and provided adequate information and/or data to support its opinion that these criteria are appropriate for data inclusion in the DB? If not, what additional information should we provide?	
REVIEWER	**COMMENT**

	• Section 2.3
	  ○ Why did you decide not to have a separate class to distinguish (non-)genotoxic compounds?
	  ▪ The carcinogenic TTC identified by Kroes et al (2004) is roughly x10 lower than the Class III TTC.
	Otherwise, the reasoning behind the selection criteria is clearly explained.

Reviewer #4	The issue of inaccurate and multiple CAS numbers, incomplete description of the chemical, etc. is described in the External Validation Database but not for the pre-validated EDT. Missing is also the extensive discussion found in the External Validation Database but not for the pre-validated EDT on the approach used when administration of the chemical was not every day or when the compound had a lower purity. For the External Validation Database there is a description of the issue of conflicting interpretations between organizations (e.g. conflicting NELs). Such issues would also have occurred but are not mentioned in the pre-validated EDT, given the number of databases used where the same chemicals are likely to have their evaluation reported. Naturally occurring substances, some endogenously produced by the body, are covered to some extent but the coverage is not complete, and the limitation of the approach is acknowledged (see answer to Q2). Unfortunately, only limited information of the selection criteria for data inclusion or exclusion in the DB is provided. Given that similar compounds can give rise to a wide range of different toxicological activities, it is also not very clear what the inclusion or exclusion criteria as to toxicological properties were. The definitions (and guidelines) for the toxicological endpoints are less clear. In part this is due to the nature, but a clear position would have been helpful. For example, the concepts of NOAEL and NOEL can be quite different. When was adversity taken into account and when where effects not necessarily linked to adversity considered? There is only reference to small increases in relative liver weight from metabolic loading, and this was excluded. Finally, there is no mention of PODs using the BMD approach. Weren't there any cases where BMDL values were used? What was the decision if both BMDL and NO(A)EL values were available? Reference is made to 'toxic effects in the context of enzyme catalyzed and uncatalyzed metabolism' (p 81). What is exactly meant with uncatalyzed metabolism? Spontaneous degradation? GSH conjugation without GST? Redox cycling?

Question 11: FDA used various factors based on study duration to derive duration adjusted no-effect-levels (NELs) to estimate chronic NELs. Has FDA provided adequate information and/or data to support its opinion that these duration adjustment factors are adequate to derive chronic NELs? If you generally agree, are there any exceptions in which these factors might be problematic to the derivation of duration adjusted NELs?	
REVIEWER	**COMMENT**
Reviewer #1	As previous two responses, it is vital that the adjustment factors are recorded transparently, as well as the process of applying them (this could be included in the aforementioned "worked example". The FDA has done a thorough and rigorous job of defining and applying the factors to derive the NEL. As before, it would be clearer if it could be summarized within a table, but this is a minor issue. Other comments: • Section 2: reference to the "Original" EDT... I found the use of the adjective "original" in the title to Section 2 quite confusing. If there will be different versions of the EDT then I would recommend referring to them as ver. 1.0, 1.1. 2.0 etc. rather than "original".
Reviewer #2	The derivation of the duration adjustment factors is discussed in Sections 2.2 and 3.3. A duration factor of 1 is used for studies > 98 days, 3 for studies 84-98 days, and 10 for studies < 84 days (Section 2.2, p. 80). The 3 distinct durations are non-conventional, but the reason is explained later (Section 3.3, p. 85). Further, the factors used by ICH Q3D, ICH Q3C(R6), ECETOC, ECHA and REACH are reviewed. The final decision for EDT to use the middle ground values and to continue with the Munro et al (1996) approach to establish the Cramer Decision Tree classes was also explained. While the EDT factors reflect a pragmatic approach, they will likely raise questions when compared to science-based principles as presented in ICH Q3C(R6) Appendix 3. Of important note is that ICH accounts for species-specific lifespan and life-stages.
Reviewer #3	I think there is adequate information provided to support the use of the adjustment factors FDA chose when adjusting the NELs for a chronic duration, especially when converting the different lengths of oral studies. Unfortunately, there didn't seem to be any information about what conversion factors were used for the non-oral studies. • Page 82, line 5 – What conversion factors (if any) were used for the non-oral studies? Please expand why a conversion factor was (not) used.
Reviewer #4	The External Validation Database but not for the pre-validated EDT has an extensive section on the approach used when administration of the chemical was not every day or when the compound had a lower purity or when administration was of shorter duration. The same level of description should be used to describe how the EDT was created. How was the situation handled where a NEL for the shorter duration study was lower than that for the chronic study? In the situation where substances were tested only in single-dose studies, it is stated that they were included if the NEL 'was within an order of magnitude as that of other members of the congeneric group in multiple dose level sub chronic or chronic studies (e.g., dimethyl disulfide)'. Would this not lead to some form of confirmatory bias?

Question 11: FDA used various factors based on study duration to derive duration adjusted no-effect-levels (NELs) to estimate chronic NELs. Has FDA provided adequate information and/or data to support its opinion that these duration adjustment factors are adequate to derive chronic NELs? If you generally agree, are there any exceptions in which these factors might be problematic to the derivation of duration adjusted NELs?	
REVIEWER	**COMMENT**
	For developmental, reproductive, or combined reproductive/developmental studies, it is stated that 'parental NEL were used in cases where the maternal or paternal NEL was less than or equal to the NEL for the offspring. Duration factors (3 or 10) were employed to adjust for the study duration for dams and males.' I could see a justification for this duration factor in the absence of any additional repeated dose study. However, if the toxicity is only manifested as a result of pregnancy than the entire duration of the pregnancy should be seen as complete, i.e. without the need for an additional duration factor. The analysis of the impact of non-oral studies is welcomed. The decision not to generate additional NELs from LELs is welcomed. It is described on p85 that 'to calculate the Class VI TTC, we decided to also use the 11 NELs from studies with a duration of less than 84 days (but no one-day studies). We used a factor of 10 to adjust for the short duration to calculate chronic DNELs, as described earlier.' While the argument to ensure maximum protection can be defended, it would be helpful to see an analysis the impact of the factor 10.

Questions for section 3 (The Preliminary (Pre-validation) Threshold of Toxicological Concern Levels):

Question 12: Based on Figure 2 and all other information provided, in your opinion, does the EDT better resolve the differing toxic potentials of chemicals with broad structural variation compared to the CDT? Please explain why or why not.	
REVIEWER	**COMMENT**
Reviewer #1	Unfortunately, I do not feel I can give a definitive answer to this question! To do this would require a greater understanding of the data, illustration of the data distribution etc., this has not been provided. It is also difficult to give a definitive answer as the functional groups etc. will be associated with a range of potencies – I agree that some are intrinsically more toxic than others e.g. replacing a primary alcohol by a nitroso group will make the molecule more toxic. However, the data distribution is dependent on the compounds that have been tested. Whilst I cannot give a definitive response, I do believe the EDT provides a much improved decision tree to support TTC analysis. The classes have been updated with contemporary knowledge and understanding, as well as newer data. This, in itself, gives me greater confidence in the use of the EDT. Comments on the data in Table 2: • The difference between the total number of substances in each class and that used for the TTC calculation should be carefully explained and the compounds omitted also included in the data (with reasons for exclusion).

Question 12: Based on Figure 2 and all other information provided, in your opinion, does the EDT better resolve the differing toxic potentials of chemicals with broad structural variation compared to the CDT? Please explain why or why not.	
REVIEWER	**COMMENT**
	• Class VI: I have no doubt FDA will be criticized that this class has a low number of substances (just as Cramer Class II in Munro has been). The reason for the low number is obvious i.e., these are unique and identifiably highly toxic compounds so not many will be tested. Whilst there may well be criticism, I would like to offer my support for this class, despite the low number of substances. • Could further analytics be added to Table 2 e.g., range of molecular weights (this should be part of the applicability domain) and even range of NELs (along with 5th / 95th percentiles to make it more realistic)? • It might be helpful to explain why molar units are used rather than mg, and how does this affect the distribution (remember since Munro et al 1996 we have traditionally used mg values) On a note of presentation. Fig 2 in the FDA review document (Overlap of NELs...). My preference would for the x-axis (log NEL) to increase in value from left to right, this is more logical when thinking of dose data and consistent with Fig 2 in Munro et al (1996). It may also be possible to indicate the 5th percentile such that the derivation of the TTC value would be apparent.
Reviewer #2	Figure 2 appears to give better resolution of the 6 EDT classes than the 3 CDT classes. EDT proposes to use mmol/kg bw/day for NEL. The rationale and literature support are explained in Section 2, p. 80. Although the large MW compounds such as proteins and polymers are out of scope, the MW range for the EDT database may still be quite wide. In addition, a single molecule may have multiple alert features and/or reactive sites on the same molecule. It would be good to compare how the proposed unit works for the more complicated molecules that exhibit multiple toxicity endpoints. Table 3 compares the pre-validation EDT TTCs with and without use of the median MW. It would be helpful to include the MW ranges in the table. High MW compounds may have been excluded for various reasons. Is there a cutoff for MW before a large complex organic compound is excluded?
Reviewer #3	Based on the information provided it does seem like the EDT better resolves the differing toxic potentials of chemicals compared to the CDT. This is because 1) the EDT Classes cover a wider range of NEL values than the three Munro classes and 2) the fact there are more EDT Classes means that the TTCs can be associated with a finer selection of chemicals with a more similar toxicity. However, there are some questions/suggestions I have that could be used to make this clearer and improve the comparison against the CDT: • How many orders of magnitude does the CDT DB data span? This comparison would be useful to highlight the extra NELs the EDT covers. • How were the number of EDT Classes chosen? • If you have the CDT DB data and you're able to recalculate the TTCs in ug/kg bw/day, it could be good to convert the NELs in the CDT DB to mmol/kg bw/day and calculate the CDT TTC values using the median MW (similar to what you did for the EDT TTC). This would give you another way to compare the results for the CDT DB to those for the EDT TTC.

Question 12: Based on Figure 2 and all other information provided, in your opinion, does the EDT better resolve the differing toxic potentials of chemicals with broad structural variation compared to the CDT? Please explain why or why not.	
REVIEWER	**COMMENT**
	• Figure 2 is a nice graphic to illustrate the additional coverage provided by the extra EDT Class and what the distribution of NELs looks like. But it is a little difficult to distinguish where the lower NELs values are, so it might not be the best to show the decreased overlap in NELs. Maybe it'd be good to use a box and whisker plot or violin plot to show that? • It might also be worth performing separate pairwise comparisons of the distributions for the CDT and EDT Classes to show whether, for example, the CDT Class I is statistically different from the EDT Class I/II. Or whether the EDT Class I is statistically different from the EDT Class II, etc. ○ This could be done using a metric like the Kolmogorov-Smirnov test. • What CDT Class are the chemicals in the example on Page 86, line 23 in? Do these chemicals fall into the same CDT Class? • This may be something for an earlier Section, but how was the default EDT Class chosen?
Reviewer #4	The steepness of the curve supports the argument for a better differentiation. However, it would have been interesting to see the EDT curves against the more recent improvements to the TTC by having a total of five groups (the three Cramer ones, the OP-carbamate one and the DNA-reactivity one). The differentiation is also helped by the fact that the EDT range is much wider than the TTC one, primarily helped by the fact that the thresholds of the two lower classes are higher than in the original TTC.

Questions for section 4 (The Validation of the Expanded decision Tree):

Question 13: Has FDA clearly explained the source of the validation DB and how the data was verified pre-validation? If not, what additional information should we provide?	
REVIEWER	**COMMENT**
Reviewer #1	The FDA indicate clearly that the validation DB was harvested from the US Environmental Protection Agency (EPA) ToxVal DB. It would be good practice to give the URL / reference for the ToxVal DB (obviously it can be found with an internet search engine, but that is not the point). Also, the version number of ToxVal DB, dates of data collection etc. should be given. The purpose of such detail is that any updates in ToxVal DB can be accounted for. The verification of data is described in Section 4.3.4. There is a really interesting and important statement here that the original study report often did not agree with ToxVal DB (as I understand what is stated). As an aside, this should be reported and the reasons for it established. Section 4.3.4 states that "our own judgement" as used to settle on the NOAEL. This is a potential area of ambiguity, I am assuming that the process is as described in Section 4.3.5, although that is not made clear. Regardless, FDA should ensure that this process is described fully (Section 4.3.5 is probably adequate) and all decisions are recorded in the database. As with my response to Question 10, this may be a place where a "worked example" would help others understand how this has

Question 13: Has FDA clearly explained the source of the validation DB and how the data was verified pre-validation? If not, what additional information should we provide?	
REVIEWER	**COMMENT**
	been done.
Reviewer #2	Section 4.2 describes the EPA ToxVal DB as the original source. By applying filters, a small subset was created with defined structures with sub-chronic and chronic oral studies and derived NELs. The processing and verification for pre-validation appears robust (Section 4.3).
Reviewer #3	• Page 88, line 4 - What version of the ToxVal DB was used? • Page 88, line 6 - What was the minimum number of days a study had to be conducted for in order to be used in the validation DB?
Reviewer #4	Contrary to the pre-validated EDT, the information on the validation DB (External Validation Database) is well described. What is missing is a clear overview of the databases used for the EDT and the External Validation Database, the number of chemicals extracted from each database and any overlap. Some tables and figures would be helpful here to guide the reader.

Question 14: Has FDA clearly laid out how the validation DB received from EPA was processed to enable its use for the external validation of the EDT? If not, please explain why not.	
REVIEWER	**COMMENT**
Reviewer #1	The FDA has clearly listed how the validation DB was processed. Some comments: • Was an IUPAC name recorded (where available)? • Which SMILES code was used? There are multiple types of SMILES, plus many ways of recording the same (e.g. toluene could be Cc1ccccc1 vs c1cc(C)ccc1 vs CC1=CC=CC=C1 etc). I would recommend a single approach is used e.g. canonical SMILES. The other option is the use of InChI / InChIKeys, which would be a good standard identifier anyway. This point is vital to identify duplicates both within the Validation DB and the EDT DB. • Section 4.3.2. Applicability domain is mentioned here but much less so for the EDT DB. There is an opportunity to properly investigate the chemical space of EDT DB and whether the Validation DB meets this. For instance, this could be achieved with Principal Component Analysis of descriptors / fingerprints, or many of the widely used machine learning techniques. • Sectio 4.3.3. Read-across data. The FDA is correct not to use read-across data. Did the data for the “source compound” of the read-across enter the same evaluation procedure as the ToxVal DB data? Data from ECHA’s DB are notoriously difficult to use, as well as having the problems of commercial sensitivity. • Typos ○ Section 4.3.1. Paragraph 1, line 4 “exists” should be “exist” ○ Section 4.3.1. Paragraph 2, line 1 delete space in “counter ion”
Reviewer #2	Section 4.3 provides details on the processing and verification of the data

Question 14: Has FDA clearly laid out how the validation DB received from EPA was processed to enable its use for the external validation of the EDT? If not, please explain why not.	
REVIEWER	**COMMENT**
	received from EPA with respect to elimination of duplicate substances, applicability domain, read-across data, selection of NEL, selection of best representative study, as well as dose and purity adjustment. The details are clear and well laid out.

Reviewer #3	• Page 88, line 20 - How was the cross-referencing performed to remove chemicals already in the EDT DB?
	  ○ What was used as the comparator, the CASRN? SMILES?
	• Page 88, line 34-Page 89, line 2 - Do you have a list of the counter ions that were removed up-front from the chemicals in the validation DB or were chemicals with different salt forms run through the EDT workflow and only removed after having an EDT Class assigned?
	• Page 89, line 12 – Were the SMILES from the EDT DB and external validation DB canonicalized using the same software before comparing them?
	• Page 90, line 40 – What did you do when different agencies had different ADIs/RfDs?
	  ○ Did you choose the lowest or use judgement to identify which you thought was more appropriate? If it was the latter, what factors did you consider?
	  ○ Page 91, lines 7-15 – What were the reasons behind this study being deemed more appropriate? The length of study, the adverse effects, it having a lower NEL?
	  ▪ Trying to get a sense of the types of criteria that were used when choosing a NEL from outside of ToxVal DB

Reviewer #4	The answer here would be similar to the one provided for Q13. See also answer to Q9

Question 15: Has FDA provided adequate information and/or data to show that the validation DB was processed appropriately for its intended use? If not, what additional information should we provide?	
REVIEWER	**COMMENT**
Reviewer #1	This question is also difficult to answer without the results, presumably which will be released as part of Phase II. It would have been helpful to clearly define what the meant by and required from the term “validation”. Validation means different things to different people. Was the purpose here to allow for regulatory acceptance? I assume not... I believe the validation process was to evaluate the performance, whether it is fit for purpose etc. As such, I think Section 4.1 should describe more fully what is intended here. I agree with and support the process of evaluation described in Section 4.1. However, it would be useful to define properly what is meant by the EDT TTCs being “protective”, I assume this means the TTC is significant below the NEL

Question 15: Has FDA provided adequate information and/or data to show that the validation DB was processed appropriately for its intended use? If not, what additional information should we provide?	
REVIEWER	**COMMENT**
	(how far below?). Section 4.1 ii) states the validation will "accurately predict the chronic oral toxicity....". Surely the purpose of TTC is not to predict toxicity, that is more like QSAR / Read-across. I appreciate these comments may be addressed by Phase II. So, I believe adequate information is provided to show the data were processed appropriately, but the problem formulation could be improved.
Reviewer #2	Please see comments to Questions 13 and 14. The FDA has provided adequate information and/or data for the pre-validation DB. Section 4.3.7 points to the additional processing of data. This additional information will be useful in Phase 2.
Reviewer #3	Yes, I believe FDA has provided adequate information to show that the validation DB was appropriately processed.
Reviewer #4	In addition to my previous answers, it would have been useful to have an overview of the types of compounds that were used to populate the EDT and the External Validation Database. By type I am referring to the principal use of the chemicals, i.e. pesticide, food flavouring, food colour, endocrine active substances, pharmacologically active substances, etc. This information would be important in the evaluation of the intended use of the EDT in the different sectors and to see that there is sufficient confidence on the applicability domains. It is also not clear how well persistent chemicals are represented and how robust the EDT is for dealing with this group of chemicals.

Phase II Peer Review Charge Questions:

Question 16: Some of the pre-validation EDT questions were updated, and some new sub- and sub-sub-questions were created based on the validation results. Has FDA provided adequate information to justify all updates? If not, which changes/updates were not fully justified and what information should we provide to justify them?	
REVIEWER	**COMMENT**
Reviewer #1	I found that the updates to the pre-validation EDT questions were well explained and justified. They gave me sufficient detail to understand why the update had been made in a clear and unambiguous manner. It is certainly impressive how much additional work, post-validation, that has been performed and how much knowledge was extracted from the whole validation procedure.
Reviewer #2	The updates to the pre-validation EDT questions are very helpful, based on the validation results. FDA shows great care to address the comments from the chemists and provide thoughtful resolutions for toxicological evaluations. Below are general comments for consideration, much of which is probably already work-in-progress for the next phase of EDT development. Further insights on the ongoing updates would be much appreciated. Below are general, high-level comments with cross-reference to other charge questions as relevant and appropriate.

Question 16: Some of the pre-validation EDT questions were updated, and some new sub- and sub-sub-questions were created based on the validation results. Has FDA provided adequate information to justify all updates? If not, which changes/updates were not fully justified and what information should we provide to justify them?	
REVIEWER	**COMMENT**
	• Appendix 1: The updates are explained in Section 4.4 and corresponding edits are tracked (red) in Section 4.5.4 for the post-validation, finalized EDT questions. Appendix 1 provides short explanations for the pre-validation EDT questions. Now that the EDT questions are finalized, it would be very helpful to integrate all the changes and update Appendix 1. [Please also see also comments to Appendix 1, Charge Questions 19 and 20] • Read-across: In Phase II, read-across is introduced as a very important scientific tool to fill data gaps for data-poor compounds in congeneric groups. Read-across has helped to greatly expand the number of compounds in the final EDT database. I am in total agreement to integrate read-across into EDT. While there is no consensus standard in the scientific or regulatory communities on read-across methodologies, please consider elaborating on the principles, key criteria and literature references (Escher et al., 2019; European Chemical Agency, 2017; Firman et al., 2021; Patlewicz et al., 2024; Patlewicz & Shah, 2023; Punt et al., 2020). With the recent advances in computational (Q)SAR modeling, it would be helpful to compare available software programs such as EPAs GenRA and ECHA's OECD Toolbox and provide transparency on the EDT read-across approach. [Please also see comment to Charge Questions 17 and 18] • Bioavailability: The EDT DB collects data on toxicokinetic and metabolic fate of substances to evaluate the influence (or lack) of absorption and metabolism on the toxicity of the substances, rather than gathering comprehensive ADME datasets (Section 2.1). It is true that absorption is among the key determinants of the bioavailability of substances. However, oral absorption does not truly represent systemic (internal) absorption. One scenario is that substances that are poorly absorbed may be rapidly eliminated to a large extent. Such substances may be biologically inert and eliminated from the body with no adverse effects. Then there is another scenario where reactive substances may be sequestered/bound in body organs/tissues, resulting in low distribution and low elimination. Therefore toxicological risk assessments would benefit from use of broader ADME understanding to predict systemic (internal) rather than oral (external) exposure dose (Partosch et al., 2015). Commercially available software programs, e.g. ACD Percepta (http://www.acdlabs.com/products/percepta/), may be useful for this purpose. [Please also see updated comment to Question 12]
Reviewer #3	I think, for the most part, FDA has provided adequate information to justify the updates based upon the validation results and feedback from the validation chemists. However, I have some minor clarifying questions: • Q6a – While the clarification on page 24, lines 38-44 make sense, did you use data from the EDT DB or references to back up this change? • Q7 – There are references to Q6b(i) and Q6b(ii) on page 27, I am assuming these are typos, but wanted to include them.

Question 16: Some of the pre-validation EDT questions were updated, and some new sub- and sub-sub-questions were created based on the validation results. Has FDA provided adequate information to justify all updates? If not, which changes/updates were not fully justified and what information should we provide to justify them?	
REVIEWER	**COMMENT**
	• Q7g – Could you add an explanation for the clarifications made to Q7g. • Q7 assignments – Could you add an explanation for the reassignment of chemicals answering yes at Q7g(iv). • Q14 assignments – Could you add an explanation for the update for moving chemicals where "…the epoxides are substituted by or fused to a polyaromatic ring…" if yes to Q14b(ii). ○ The pre-validation EDT assigned polyepoxides to Class V, rather than moving them onto Q33. In Appendix 2, the results for the 4 chemicals answering yes to Q14b(ii) also seem to suggest they were not moved onto Q33.
Reviewer #4	This reviewer submitted a multi-page combined comment instead of individual feedback for the phase 2 questions 16-22. Please see this response at the end of this section.

Question 17: Was the validation adequate to show that the EDT is suitable for the classification of compounds in its applicability domain according to their toxic potentials? If not, describe what type of validation would be needed.	
REVIEWER	**COMMENT**
Reviewer #1	As there is no advice to the contrary, I take the term "applicability domain" to be as it is used with regard to a QSAR. That is the applicability domain is a representation of the chemical and structural property space, including metabolism and, where possible toxicology and mechanism of action. Therefore, I take this question to be asking whether the domains for the six classes have been assessed with the validation exercise. My personal opinion is that the validation exercise was an extremely useful and valuable activity, but it has shown little about the applicability domains within the EDT. By this I mean that the applicability domains within the EDT were not defined as I would like and as a user would find helpful. For instance, it would be helpful for each of the six classes to be summarised in a figure with the types of chemistry that are associated with them. This is obviously cross referenced against the questions. Further the applicability domain could give an indication of the range of physicochemical properties, for instance log P, molecular weight etc for the compounds associated with them in the supporting database. Hopefully the software that is being developed could have this type of functionality, so that a user would be able to know, at least in broad terms, whether their compound falls within the domain of the EDT. In terms of the validation exercise that could be undertaken, the prevalidation database could be defined as described above, with regard to structural features and properties, and the validation database mapped onto those properties and features for each of the six classes.
Reviewer #2	The finalized external validation DB contains 1,242 substances bringing the

Question 17: Was the validation adequate to show that the EDT is suitable for the classification of compounds in its applicability domain according to their toxic potentials? If not, describe what type of validation would be needed.	
REVIEWER	**COMMENT**
	Combined EDT DB to 3142 substances (Appendix 2). In order to evaluate whether they are suitable for their classification in the applicability domain, it would be important to clearly define the EDT DB domain and describe the criteria and validation for QSAR modeling purposes. In Section 4.3.2, there is only mention of compounds eliminated when not in the applicability domain: "unhyrolyzable polymers, proteins, inorganic substances, and substances with undefined structures in addition to most mixtures". In Section 4.6.1 and Section 4.6.2, there is intention to broaden the applicability domain but without explicitly defining the domain. A recent publication by Mora et al (2024) describing applicability domain analysis in QSAR models may be helpful. For the EDT DB domain, it would be helpful to identify the compounds by their intended uses, e.g. as direct/indirect food additives, monomers, plasticizers. In addition, many of the substances are identified as naturally occurring, e.g. plant flavonoids (Appendix 1 explanation on Charge Question 15). Identifying naturally occurring substances would help assess the applicability domain of the EDT DB for the dietary intake of the general population as well as the special populations at risk. The suggestion is not to burden the EDT DB with naturally occurring substances of low order of toxicity. Rather it is to understand whether the EDT DB includes naturally occurring substances with known significant bioactivity and potential toxicity. ClassyFire is a freely accessible program (http://classyfire.wishartlab.com/) that classifies chemicals into structure-based taxonomy (Djoumbou Feunang et al., 2016). It may expedite classification of the substances in final EDT DB into chemical families and/or congeneric groups and provide further support for applicability domain, as well as read-across approach.
Reviewer #3	Yes, I think the validation is adequate to show the suitability of the EDT. The use of the pre-validation EDT questions to classify the validation chemicals by the validation chemists helped to identify areas of the workflow that were not covered by the chemicals in the pre-validation DB. I believe combining this with the evaluation of the EDT Classes after the assignment of the validation chemicals was a necessary step in the validation of the EDT to ensure that chemicals were not being erroneously assigned to Classes that were too under/overprotective of the chemicals toxic potential. Together these aspects of the validation allowed for clarification and/or updating the EDT questions to cover the (slightly) broader range of chemicals present in the combined EDT DB. There are a couple of analyses that I think may help compare the pre- and post-validation EDTs: • Figure 4 compares the NEL distributions for the combined EDT DB chemicals, what do the distributions of the NELs in the pre- and post-validation EDT look like for the subset of chemicals in the original EDT DB? ○ This would provide a good "apples-to-apples" comparison between the pre- and post-validation EDTs.

Question 17: Was the validation adequate to show that the EDT is suitable for the classification of compounds in its applicability domain according to their toxic potentials? If not, describe what type of validation would be needed.	
REVIEWER	**COMMENT**
	• Even though the NEL distributions look different visually, did you use any statistical method(s) (e.g., Kolmogorov-Smirnov [K-S] test) to verify that the distributions between the post-validation EDT Classes are different? ○ This could also be performed to compare the pre- and post-validation EDT Classes • Even though the pre- and post-validation EDT TTCs are very similar, you could use a bootstrapping approach to compare the 5th percentile values for the pre- and post-validation EDT to show they are not statistically different.
Reviewer #4	This reviewer submitted a multi-page combined comment instead of individual feedback for the phase 2 questions 16-22. Please see this response at the end of this section.

Question for section 5 (Conclusions):

Question 18: Has FDA provided adequate information and/or data to support the conclusions found in this section? If not, what additional information should we provide?	
REVIEWER	**COMMENT**
Reviewer #1	A number of broad conclusions are given in Section 5. I believe the report in its entirety has provided adequate information to support the conclusions made in Section 5, with the possible exception of read across, as noted below. • I agree on the hypothesis that structurally or metabolically similar chemicals can be expected to be toxicologically similar. I agree that the FDA has made a laudable effort and great improvement in the capability of grouping chemicals, establishing trends and using the TTC approach in chemical safety assessment. I also agree that the groupings are based on a large and reliable database of publicly available toxicology data. However, I find it more difficult to support the conclusion that there were many ADME data considered (see, for instance the middle of the first paragraph of the conclusions). • At the end of the first paragraph reference is made to performing read-across. I accept that the knowledge within the EDT, particularly the structural groupings, will be of great benefit to performing read across and I am excited by this prospect. However, this is a topic that is not developed in the report so I cannot agree that FDA has provided adequate information to support this particular conclusion - whilst I do accept it is valid. • I can accept in the second paragraph the statement about the level of data available and that the EDT shown that further data can be added in to improve and expand the analysis. I fully support the conclusion that periodic review will help reaffirm and strengthen the scientific basis of the EDT, and I hope that this will be possible and undertaken in the future by FDA or others.

Question 18: Has FDA provided adequate information and/or data to support the conclusions found in this section? If not, what additional information should we provide?	
REVIEWER	**COMMENT**
	• I also agree with the third paragraph that the EDT will be an invaluable tool for application with TTC and I anticipate that this will become the industry standard and then their future. • Finally, I can support the conclusion that the EDT, as part of a tiered strategy or an initiative such as Next Generation Risk Assessment, will be a valued part of reducing animal use in, and the cost of, chemical safety assessment.
Reviewer #2	The EDT project is a monumental undertaking and reaching Phase 2 is a significant milestone. The conclusion acknowledges that as additional information and data become available, the EDT can be further refined. The current conclusions are based on the premise that substances of similar structures are expected to have similar metabolic fate and to lead to similar toxicological outcome. However there is desire for deeper understanding and interpretation of more comprehensive ADME datasets, particularly how oral absorption translates to systemic bioavailability. Given that the oral studies make up about 96% of all the studies in the EDT DB, it would be of great value to the scientific community if EDT DB can relate to other exposure routes by modeling bioavailability and can provide guidance on route-to-route extrapolation factors. FDA considers EDT as a tool for toxicologists to predict toxic potentials of compounds and provide safe intake levels (TTC). The sequence of chemical structure-based yes/no questions leads to the assignment of the substance to one of the 6 classes of TTC. These questions may be intuitive to a chemist but they are difficult for a toxicologist to follow. However, we are encouraged that the EDT software is currently in development to automate read-across. We look forward to the next phase of collaboration between the chemists and the toxicologists for a user-friendly automated computer program. As the applicability domain grows with additional external data, the EDT DB will be periodically reviewed, assessed and refined. The scientific community would certainly welcome broadening of the domain and the transparency to the ongoing data collection and nomination process.
Reviewer #3	The basis on which the EDT was created is scientifically sound (i.e., structurally similar chemicals are [expected to be] toxicological/metabolically similar). The EDT questions and the workflow as a whole are structured in a way that enables the chemical groupings to be of use for read-across. The validation effort that was undertaken also illustrates that when additional data are available the EDT can be refined to classify chemicals into a more appropriate class.
Reviewer #4	This reviewer submitted a multi-page combined comment instead of individual feedback for the phase 2 questions 16-22. Please see this response at the end of this section.

Appendix 1 aims at providing a brief explanation of each EDT question. By no means are these explanations meant to be comprehensive. With that in mind, please respond to the following questions.

Question 19: Are all explanations clear and concise? If not, please identify the explanation by question number and elaborate as to how we can more clearly explain the question.

REVIEWER	COMMENT
Reviewer #1	I am basing my response to Questions 19 and 20 with regard to Appendix 1 that was provided in the first document for review (Phase I). It is noted that this has not been updated post validation. I apologise if I have this incorrect. I found the explanations in Appendix 1 clear and correct. I believe that in combination with the details on the questions, these will be very valuable for users of the EDT.
Reviewer #2	Appendix 1 gives a detailed description of the compounds and the associated mechanisms of action and toxicity endpoints (e.g. cardiovascular toxicity, neurotoxicity, carcinogenicity, oxidative stress). The terminal question, Question 28, was updated but it is still confusing (to a toxicologist). It would be helpful to provide the basis for this terminal question, sub-questions i), ii), iii), iv) and v) in ranking these toxicity endpoints into Classes III, IV or V. For example, is weighting applied to the endpoints to account for severity leading to different Class assignments?
Reviewer #3	Given the complexity of what some of the questions are trying to identify and group, I think the explanations are clear and concise. One possible way to make the explanations more easily understood may be to have them in the same order they appear in the questions. However, this could make them less concise because it may involve some repetition; for example, Q7 would involve splitting the explanation of the bioactivation of chemicals via GHS [captured by Q7g(i)] from the explanation of detoxification of chemicals via GHS [captured by Q7b(iii)].
Reviewer #4	This reviewer submitted a multi-page combined comment instead of individual feedback for the phase 2 questions 16-22. Please see this response at the end of this section.

Question 20: Should FDA add anything to these explanations to improve the reader's understanding of each question's rationale? If yes, please identify the explanation by question number and explain how we should revise. Please note that these explanations were designed to be concise and not all-encompassing.

REVIEWER	COMMENT
Reviewer #1	I found Appendix 1 to be clear and will aid the reader's understanding. They are written at the correct level, i.e. a toxicologist (or risk assessor) with a knowledge of physiology, biochemistry and chemistry. They are well referenced and with adequate evidence. As noted in response to a previous question, I hope that it will be possible to combine the information in Appendix 1 with the questions in some way,

Question 20: Should FDA add anything to these explanations to improve the reader's understanding of each question's rationale? If yes, please identify the explanation by question number and explain how we should revise. Please note that these explanations were designed to be concise and not all-encompassing.	
REVIEWER	**COMMENT**
	hopefully in the software, such that the user can see both together. My only comment with regard to making Appendix 1 clearer for the reader would be to add a title to each question, summarizing and describing the individual EDT questions. Referring to the previous question on applicability domain – aspects of this could be summarized here, as well as exemplar compounds with data. It would also be good to have an indication of how many compounds in the database were classified according to the EDT question.
Reviewer #2	Integrating the updates in Section 4.4 and corresponding edits (Section 4.5.4) into Appendix 1 would be crucial for fuller understanding and feedback to the explanations. Question 28 is the critical terminal decision question that classifies substances into EDT Classes III, IV or V. Please consider additional details on the weighting and ranking of the toxicity endpoints that differentiate these higher classes.
Reviewer #3	I think where there is some explanation of the reaction mechanism by which groups of chemicals may become (de)toxified [e.g., Qs 7b(ii), 19b, 19d, 32a, 33, and 38] to include an example illustration of what the reaction mechanism looks like.
Reviewer #4	This reviewer submitted a multi-page combined comment instead of individual feedback for the phase 2 questions 16-22. Please see this response at the end of this section.

Appendix 2 contains the combined, finalized EDT Chemistry, Toxicology and Metabolism DB on which the finalized TTCs were based.

Question 21: Are the set of chemicals in the database sufficient to cover the chemical domain of applicability described in the document? If not, please explain.	
REVIEWER	**COMMENT**
Reviewer #1	This is a very difficult question to answer. The FDA has assembled a very large database of toxicological information, having carefully curated the values. This, in itself, is a great achievement. I am assuming from the question that the "chemical domain of applicability" described in the document relates to the EDT questions. In order to address this question, I would need to see a breakdown of how many compounds were associated with each question and each assignment of a class (many EDT questions have multiple classes). In order to understand the applicability domain of the classes, I refer to Table 8 (pages 131-132) in the Phase II document. With the exception of Class VI, there are a significant number of data points in each class. Without analytics, e.g. structural features, ranges of properties etc., it is not possible to give a definitive answer with regard to Classes I to V. Class VI is quite unique and, as noted in the report, it has limited numbers of compounds due to the high toxicity associated with this class. I have no doubt of the need of Class VI and its validity. Despite the small number of data, I am satisfied Class VI is relevant

Question 21: Are the set of chemicals in the database sufficient to cover the chemical domain of applicability described in the document? If not, please explain.	
REVIEWER	**COMMENT**
	and significant.
Reviewer #2	Question 21 (for Appendix 2) and Question 17 (for Section 4) both relate to suitability/adequacy of the chemical and applicability domain of EDT DB. Specifically for Appendix 2 (spreadsheet), please consider: • Adding MW unit to Column E (i.e. µg/kg bw/day or mmol/kg bw/day) • Add footnote or text to explain Column E on how molecular weight is adjusted based on # of subunits. It is not clear if subunits refer to repeating units as in polymers and/or copolymers. • Adding column for intended/approved use of the compound (e.g. direct food additive, indirect food additive, polymer production, monomer, plasticizer, catalyst, cross-linking agent, curing agent) (Sheftel, 2000) • Adding column for Chemical congeneric group/class/family for each compound • Adding column for critical toxicity endpoint(s) on which the EDT class is based Question 21 does not specifically ask the reviewers to critique the critical studies selected for the derivation of NELs or LELs. Perhaps this is planned when new and significant studies are published.
Reviewer #3	Yes, I believe the chemicals in the combined EDT DB are sufficient to cover the chemical domain of applicability described.
Reviewer #4	This reviewer submitted a multi-page combined comment instead of individual feedback for the phase 2 questions 16-22. Please see this response at the end of this section.

Overall question:

Question 22: Do you have any other comments or suggestions?	
REVIEWER	**COMMENT**
Reviewer #1	I fully acknowledge the work that has been undertaken to create the EDT, its validation and the preparation of the database. I understand that it has been many years in the making, and the complexity and level of detail as well as coverage across chemical classes is testament to the hard work of the FDA. I look forward to the publication(s) that will arise from the EDT and encourage FDA to include as much detail as possible. This is particularly the case because we know from the original Cramer scheme there was much ambiguity. This is, of course, not a criticism of the original scheme which was quite revolutionary in its time. I also hope that FDA will promote the scheme, e.g., at conferences, webinars, social media etc. There will also be a need for training in the new scheme and this in itself is an opportunity for dissemination and to increase understanding in the whole concept of the EDT and TTC. The “make or break” for the EDT will be the software platform on which it is based. I really look forward to seeing this and I hope there is an opportunity to start with a clean slate, rather than seeing it as an update of the implementations of the Cramer scheme. I hope the informatics implementation will be robust and representative of the chemistry as described in the EDT.

Question 22: Do you have any other comments or suggestions?	
REVIEWER	**COMMENT**
	Anyone who has used the implementations of the Cramer scheme well understand the limitations and ambiguities. I would also encourage the FDA to link the EDT to the data in the database, as well as provide a full description of the classes and questions, investigating property and chemical structure space - there is an opportunity here to implement some analyses with, for instance, the ToxPrint fingerprints and assessment of chemical / property space. I realise, however, that a wish list for the software is going beyond the remit of the charge questions! One further comment: Phase II report. Page 17. Section 4.4.3.20. I assume the reference to Appendix 3 should be Appendix 2?
Reviewer #2	It is quite a humbling experience for a toxicologist to read through the rationale, the thought processes and actual structure-based decision steps that help transform CDT to EDT. Toxtree, the software based on CDT, has been the pragmatic solution to predict and screen toxicity potential of data-poor compounds and to classify them into Cramer Classes I-III to estimate the corresponding Cramer TTCs. The desire for EDT to broaden the chemical domain and to advance the structure-based framework is admirable. In Section 4.6.3, it is stated "Going forward, the finalized EDT TTCs will be used." The process to achieve consensus with the scientific and regulatory communities is of utmost importance to share the EDT framework, scientific rationale, the derivation of the EDT Classes and TTCs through workshops and conferences on the ongoing transformation efforts.

Reviewer #3	Along with the updates suggested above I have some clarifying questions on the validated EDT questions/comments:
	• Throughout – I think the questions would be easier to read if the additional context in parentheses were included using square brackets, saving the parentheses for the question numbers and SMARTS patterns.
	  ○ For example, Q3g(viii)) is an example where it is especially unclear what is (not) meant to be in parentheses.
	• Throughout – I think the questions would be clearer if each sub-sub question were on its own line
	• Section 4.4.3.56 – page 38, line 20, should "alicyclic block" be "acyclic block"
	  ○ It appears that Q24a and Q24b are for acyclic chemicals
	• Section 4.4.3.57 – page 39, line 14, should this be Q33c rather than Q43c?
	• Section 4.4.3.58 – page 40, lines 9, 11, and 14; should this be Q34a(i))/Q34a(iii)) rather than Q43a(i))/Q43a(iii))?
	• Definition Q – Page 51, line 12, "see definition AA" should be updated to definition Z.
	• Q1c – Is the exemption of ≥8 contiguous conjugated double bonds [Q1a(iii)] only applicable to Q1c(i) or all of Q1c?
	  ○ Page 6, line 5 seems to suggest it is all of Q1c, but the question itself reads as it is only Q1c(i).
	• Q2e – Can the -OH group(s) on the alkyl chain be directly attached to the P?
	• Q2e – Can both -OH and the ester/N be on the same alkyl chain?
	• Q2 assignments – Should iv) also check if yes to Q2a)?

Question 22: Do you have any other comments or suggestions?	
REVIEWER	**COMMENT**
	○ Should it read "If yes to a) and e(i)) or e(ii)) assign to Class II? • Q2 assignments – Should v) also check for e(i)) and e(ii))? • Q3c(ii)) – It would be good to use the statement "…(but these cannot be a part of a heterocyclic ring itself)…" here to be consistent with Q3c(i)). • Q3d – Does the definition of "connector" on Page 9, line 28-29 need to be added here or with the other definitions? • Q3f(iii)) – Does it matter if the N+ is directly or indirectly substituted? • Q3f(vi) – There are some typos here "…a maximum or 2"; comma between "one or more" and "ether, alcohol, ester…", and; "single heteroaromatic rings" • Q3f(vii)) – The term "…only one ring with a single ring N+…" may be easier to understand as "…one ring with a single N+ as a ring atom…" to keep consistent with how it is in other questions • Q3g(ii)) – Page 69, line 1: May be good to add "[i.e., completely contained within a ring]" after "derivatives can be fully cyclic". • Q3g(viii)) – I think the closing parenthesis may be missing. • Q5 assignments – The "Examples for no" heading either needs to be reworded or new examples should be included. The current examples (methylmercury and darinaparsin) are now examples of chemicals that contain atoms in points (i) and (ii) rather than examples for no. • Q6e(iii) – Should the "Examples for Q3e(iii)" be "Examples for Q6e(iii)"? • Q6h(iii) examples – It would be clearer if the example ring structures started on a new line ○ I think "cains" is a typo and should be "chains" • Q18b examples – I am assuming the substituents of interest are currently in blue and the rest of the structure is in red because it is an addition to the post-validation EDT. I would recommend having the substituted associated with "yes" assignment in red in the final EDT documentation. • Q28n(ii)) – The text in parentheses "(other than in the previous sub-sub-question)", may be better written as "(other than those captured in sub-sub Q28n(i)))" so that it is more inline with similar text in Q18a(ii)). • Q36c – Was "linear aliphatic chain of ≤6" a typo in the pre-validation EDT? Is that why it was updated in the post-validation EDT.
Reviewer #4	This reviewer submitted a multi-page combined comment instead of individual feedback for the phase 2 questions 16-22. Please see this response at the end of this section.

Questions 16-22	
REVIEWER	**COMMENT**
Reviewer #4	**1. General Impressions** The idea of using thresholds of exposure to support regulatory decision making was first formulated in the 1960s and further developed into the concepts of virtual safe doses and the Threshold of Regulation (for exposure to potential carcinogens) and into Munro's TTC approach in the 1990s (for non-cancer

Questions 16-22	
REVIEWER	**COMMENT**
	endpoints). The original TTC was derived from a database of 613 chemicals that were allocated into one of three so-called Cramer classes based on structural considerations. Over the subsequent decades, the TTC approach was further refined, primarily by the inclusion of additional chemicals, adjustments of some of the Cramer questions, the reassignment of some groups of chemicals, the definition of exclusion criteria, and perhaps the most impactful of all, the definition of additional threshold values for organophosphates and carbamates, and for compounds with structural alerts for genotoxicity. However, it is fair to say that the impact of extending the chemical database has been seen mainly as confirmatory for the approach, and that in practice the three Cramer class thresholds have remained unchanged. Therefore, while the TTC approach has been widely acknowledged as a useful screening and prioritization tool for the risk assessment of chemicals when hazard data are incomplete, concerns always remained as to its coverage of the chemical space because of its reliance on just over 600 chemicals. There was also concern over the small number of chemicals used to derive the Cramer Class II threshold value. For these reasons, the initiative of FDA to engage in a complete re-assessment of the Cramer et al. Decision Tree (CDT) approach using not only a much-extended chemical database (DB) of 3142 different chemicals but also by developing from scratch a set of questions for the decision tree approach is very welcome. The project sent out for the reviewing process was divided into two phases. The first phase consisted in the review of the original Expanded Decision Tree (EDT) followed by its external validation. This phase included the charge questions. Phase II focused on the validation of the EDT and presentation of the outcome of the finalised post-validation. In the finalised post-validated EDT, many of the EDT questions were refined or expanded by introducing sub-questions to avoid misclassifications (too conservative or insufficiently protective, dead-ends or oversight of groups of chemicals or structures. Reviewing EDT Phase II was facilitated by the provision of the final EDT DB which allowed this reviewer to test the EDT questions and compare with the class assignments reported in the DB. The lack of access to the final DB rendered some aspects of the review of Phase I challenging. **2. Detailed Comments** The EDT enables the assignment of chemicals into one of six EDT classes. A clear advantage over the original Munro TTC is the much-reduced overlap between classes as compared with the Cramer classes plus a wider range between the Class I and the Class VI thresholds. It is, however, difficult to compare the distribution of the EDT classes with the distribution of the organophosphate-carbamate and the DNA-reactive classes of chemicals. Of the 3142 chemicals found distributed in EDT DB, 2057 were used to derive the six threshold values. From the description provided, it is not very clear what inclusion/exclusion criteria were used to determine which compounds contributed to calculate the TTC values and which did not. It would have been useful and more transparent to show in the Appendix 2 spreadsheet (EDT DB) which compounds contributed to the TTC calculation. Also, useful would have

Questions 16-22	
REVIEWER	**COMMENT**
	been an indication in the spreadsheet which question (or group of questions) within the EDT were used to assign the class for each compound. Some of the questions regarding the choice of endpoint and extrapolations used to define the point of departure values were raised in the answers to the charge questions. The final EDT contains 47 main questions, most of which lead to several sub-questions. These appear to have gone through a rigorous assessment to ensure that any permutation in chemical structure can be either assigned to an EDT class or be forwarded to another question. The EDT is also aimed at supporting read-across. Here, future guidance will be needed to see how the EDT can be incorporated in a read-across framework, but the intention is welcome. Overall, the number of chemicals per EDT class appears sufficiently large to support the six classes. However, EDT Class VI is limited 68 unique chemicals, corresponding to only 3% of the total number of substances. Where the approach used to define the EDT questions becomes more problematic is when the questions focus on chemicals that have pharmacological activity and where this pharmacological activity is responsible for the toxicity of the compound or group of compounds. The EDT addresses well the poly-halogenated compounds that fall in the dioxin group and that operate through the Ah receptor. This is a well-characterised group of compounds, and the existing TEFs have helped to distribute the members of the dioxin-like chemicals into their appropriate classes. For the phthalates, the evidence for lower potency to induce developmental effects in the shorter phthalates is perhaps not sufficiently strong to have them in Class III as compared to Class IV (e.g. DEP, a suspected ED). Sometimes, it is not clear why some closely related compounds are assigned to different classes while their points of departure would not support this. For instance, MEHP is Class III although its LEL is lower than that of its parent DEHP in Class IV. Question 6 addresses, among others, structures known to act through oestrogen receptors (e.g. diethylstilbestrol and tamoxifen). Among these, there will be structural analogues devoid of or with much reduced pharmacological activity, and where a read-across will lead to an over-protective classification. Information on such analogues may not necessarily be available in the public domain. In theory, the opposite may also occur where future structural analogues may show much enhanced potency and where the EDT class may not be sufficiently protective. In the modification of the original TTC approach introduced by Kroes et al., many of these compounds were excluded, and while this reviewer welcomes the effort to minimise the exclusion categories from the EDT, for those chemicals that are toxic by virtue of their pharmacological properties, the level of uncertainty from using simple structural criteria and chemical read-across may be quite high. However, since the class assignment is generally based on the most potent members, the EDT approach would be expected to be sufficiently protective for the group of interest. The threshold for Class VI is approximately 5-fold lower than Kroes' threshold for potential DNA-reactive mutagens and/or carcinogens. Unlike for the latter, the EDT DB does not exclude the so-called cohort of concern (COC) for which it was concluded that the TTC of 0.0025 µg per kg bw per day would not be

Questions 16-22	
REVIEWER	**COMMENT**
	sufficiently protective. The question is whether Class VI would be sufficiently protective for the members of the COC. It is also noted that in the EDT DB, the nitrosamine DEN, a member of the COC, is classified as Class V which has a TTC value that is higher than Kroes' threshold for potential DNA-reactive mutagens and/or carcinogens. Another chemical that was excluded from the TTC approach by Kroes et al. is TCDD. The EDT places TCDD into Class VI yet both the TWI established by EFSA in 2017 and the PTMI established by the JECFSA in 2002 are much lower than the equivalent Class VI TTC value. Based on these conclusions, Class VI may not be sufficiently protective for dioxins and dioxin-like compounds. Alternatively, this group of compounds could be removed from the EDT TTC as members of an exclusion category. In Question 1i, the instructions are to disregard "the following commonly encountered and relatively nontoxic or of low toxicity i) metal counterions: sodium, potassium, calcium, magnesium, barium, aluminum, titanium, zinc, manganese, copper, iron, and bismuth". The relatively high EDT Class I TTC value may become problematic for counterions like aluminium, manganese and copper as their health-based guidance values could be exceeded. In addition, the formation of complexes with chemicals falling in Class I may lead to enhanced bioavailability of the metal counterion. Question 1 also deals with nucleotides, nucleosides, phospholipids, monophosphates of amino acids, and their hydrolysis products, to place them into Class I. A number of such compounds may not have been captured by Question 1. For example, the inositol derivative phytic acid is classified as III. It has very low bioavailability in non-ruminants and needs to be synthesised by our bodies. It could be considered as a hydrolysis product of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) (after further phosphorylation of IP4). Similarly, CDP-choline is in Class I but choline Cl (its hydrolysis product) is in Class II, and phosphatidyl ethanolamine is not listed but ethanolamine is in Class II. Question 6d aims at capturing naturally occurring toxins, and structural criteria are provided. However, quite a number of such toxins are left out. Given their structural complexity it is difficult to see where they would end up if run through the EDT questions. Just some examples from the marine biotoxin groups: Okadaic acid and dynophysistoxins, TTX and saxitoxins, palytoxins. Another challenge is that for many naturally occurring toxins there are often multiple variants. For instance, there are 12 known ciguatoxins found in Caribbean and tropical Atlantic waters and 29 reported ciguatoxins in Pacific waters but likely to be with different potencies, i.e. different TEFs. It is also difficult to see whether the main driver for Question 6d is chemistry or the pharmacology responsible for the toxicity of the compounds. For instance, the highly potent brevetoxins and ciguatoxins are given as example and classification under cat VI is justified. However, for the structurally very similar yessetoxins (MoA currently unknown but not acting through Navs), which are much less potent biotoxins, Class VI would be overprotective. The chemical bisphenol S would be redirected from Q 36 to Q 41. No clear indication of further redirection is given and under Q 41, BPS would be assigned to Class III. In the EDT DB it is assigned to Class II.

Questions 16-22	
REVIEWER	**COMMENT**
	3. Conclusions In conclusion, the developers of the EDT TTC are to be congratulated. The EDT TTC is a major improvement over the original Munro TTC and over its subsequent improvements. A major achievement is that the EDT TTC is based on an extensive DB that covers most existing regulatory assessments that are available and that it extends considerably the chemical space on which the TTC is based. This should have a positive impact on the confidence in the TTC approach for regulatory decision making. Several issues remain that would need addressing. The inclusion of questions aimed at identifying pharmacological properties or at grouping of substances based on such properties is welcome but comes with its own challenges. However, it is clear that an EDT based exclusively on chemical determinants would have been limiting and would rapidly face similar problems as read-across based exclusively on chemical similarity, and for which now an important focus is on incorporating metabolism and biological similarity. The EDT does not use exclusion categories. It is assumed that the EDT will not be applicable to nanomaterials and radioisotopes but among the organic chemicals addressed, some remain problematic, and it is not clear whether the Classes V and VI are sufficiently protective for potent genotoxic carcinogens such as aflatoxins and nitrosamines or non-genotoxic Ah receptor agonists such as TCDD. With some of the charged compounds that carry a metal counterion, the counterion may be more problematic than the organic molecule at the class threshold. Inclusion of naturally occurring toxins such as mycotoxins and marine biotoxins is welcome but the toxin space covered is currently somewhat incomplete. Finally, one of the strengths of the Munro TTC is that it is supported by software platforms that help the risk assessor to assign a chemical to its appropriate Cramer Class. The development of a software platform to support the EDT is mentioned in the document, and the availability of such software will be extremely important, not only to support risk assessors to help them navigate through the complexity of the EDT questions but also to help the acceptance of the EDT TTC by the global community of risk assessors.