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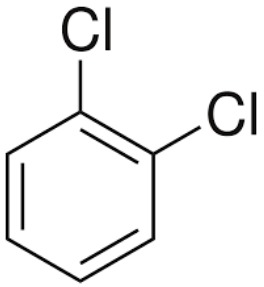
Draft Transcriptomic Assessment for *o*-Dichlorobenzene and *p*-Dichlorobenzene

Technical Support Document for the Draft Risk Evaluations: Supporting Hazard Characterization of *o*-Dichlorobenzene and *p*-Dichlorobenzene Using 5-Day *in Vivo* Transcriptomic Studies



***o*-Dichlorobenzene**
CASRN 95-50-1

***p*-Dichlorobenzene**
CASRN 106-46-7

April 2026

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KEY ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of variance
BMD	Benchmark dose
BMDL	Benchmark dose lower confidence bound
CAR	Constitutive androstane receptor
CASRN	Chemical Abstracts Service Registry Number
CPM	Counts per million
DEGs	Differentially Expressed Genes
DESeq2	Differential Expression Analysis for Sequence Count Data 2
DNA	Deoxyribonucleic Acid
DTXSID	DSSTox Substance Identifier
DTT	National Institute of Environmental Health Sciences Division of Translational Toxicology
EPA	Environmental Protection Agency (U.S.)
ETAP	EPA Transcriptomic Assessment Product
FDR	False discovery rate
GO	Gene ontology
GOBP	Gene ontology biological process
HTTr	High-throughput transcriptomics
IUPAC	International Union of Pure and Applied Chemistry
KE	Key Event
MIE	Molecular initiating event
MOA	Mode of action
MSigDB	Molecular Signature Database
NAMS	New Approach Methods
NIEHS	National Institute of Environmental Health Sciences
NTP	National Toxicology Program

OCSPP	Office of Chemical Safety and Pollution Prevention
o-dichlorobenzene	1,2-Dichlorobenzene
OPPT	Office of Pollution Prevention and Toxics (EPA)
PCA	Principal Component Analysis
p-dichlorobenzene	1,4-Dichlorobenzene
POD	Point of departure
PFAS	Per- and polyfluoroalkyl substances
QC	Quality control
RASD	Risk Assessment Support Division
SMILES	Simplified Molecular-Input Line-Entry System
TempO-Seq	Templated Oligo with Sequencing Readout
tPOD	Transcriptomic point of departure
TRV	Transcriptomic reference value
U.S.	United States

GLOSSARY OF SELECT TERMS

Term	Definition*
2-year rodent bioassay	A standardized method of testing carcinogenic potential of chemicals to humans by exposing large groups (≥ 50) of rats or mice to a chemical for 2 years, followed by a complete necropsy and comprehensive microscopic evaluation of tissues by a board-certified veterinary pathologist (OECD 453/OCSPP 870.4300).
5-day *in vivo* transcriptomic study	Exposure to a chemical in whole, living test organisms for 5 days with repeated daily dosing, at the conclusion of which tissue samples are collected from each organism for transcriptomic analysis to characterize gene expression changes after chemical exposure.
Accumulation plot	A plot quantifying the cumulative number of gene sets (*e.g.,* Gene Ontology Biological Process classes, MSigDB gene set) identified as a function of increasing benchmark dose value (*e.g.,* benchmark dose or benchmark dose lower bound).
Adverse Outcome Pathways (AOP)	An adverse outcome pathway describes a series of linked events at different levels of biological organization (e.g. cell, tissue, organ) that lead to an adverse health effect in an organism following exposure to a stressor (e.g. chemical). https://www.epa.gov/chemical-research/adverse-outcome-pathways
Analysis of variance (ANOVA)	A statistical technique that isolates and assesses the contribution of categorical factors to variation in the mean of a continuous outcome variable. The data are divided into categories based on their values for each of the independent variables, and the differences between the mean outcome values of these categories are tested for statistical significance.
Apical Effects	Observable, adverse outcomes in a whole organism that signal a toxic substance's harmful impact (*e.g.,* disease, death, etc.)
Apoptosis	A type of highly-regulated programmed cell death in which a series of molecular steps in a cell leads to its death. This is an organism's innate method of disposing of damaged, unwanted, or unneeded cells.

Benchmark dose (BMD)	A dose or concentration that produces a predetermined change in response rate of an adverse effect (called the benchmark response or BMR) compared to background.
Benchmark dose (BMD) median	The median BMD (*i.e.,* a dose or concentration that produces a predetermined change in response rate of an adverse effect (called the benchmark response or BMR) compared to background) of all genes that met inclusion criteria within a given pathway/gene set.
Benchmark dose lower bound (BMDL)	A statistical lower confidence limit on the dose or concentration at the benchmark dose.
Benchmark dose lower bound (BMDL) 5th percentile (%ile) median	The 5th percentile (statistical value indicating greater than or equal to 5% of data points in the set) of median BMDL (*i.e.,* a statistical lower confidence limit on the dose or concentration at the benchmark dose) median of all MSigDB pathways/gene sets within each superset or the combined gene set consisting of any term within the five supersets.
Benchmark dose lower bound (BMDL) median	The median BMDL (*i.e.,* a statistical lower confidence limit on the dose or concentration at the benchmark dose) of all genes that met inclusion criteria within a given pathway.
Benchmark dose lower bound (BMDL) lowest [minimum] median	The single MSigDB pathway/gene set with the lowest (*i.e.,* minimum) BMDL median within each superset or the combined gene set consisting of any term within the five supersets.
Benchmark dose (BMD) [response] modeling	Fitting dose-response data to a collection of parameterized equations (models), followed by choosing the model that best describes the data while minimizing complexity (*i.e.,* the best fit model).
Bioactivity	Ability to induce a biological effect (*e.g.,* binding to a receptor, stimulating cell growth, changes in gene expression) (ICH, 2000).
Biomarker	A molecular or cellular indicator (or "marker") of an event or condition (exposure, effect, susceptibility) in a biological system or sample. It is the product of an interaction between a contaminant and some target molecule or cell.
Concentration-responsive genes	Genes exhibiting statistically significant dose dependent expression changes as a result of the BMDExpress2 Williams' Trend Test (Phillips et al., 2019).
Constitutive Androstane Receptor (CAR)	Encoded by the NR1I3 gene, this is a nuclear receptor that regulates the expression of metabolism and excretion genes in response to endobiotic or xenobiotic substances, playing a major role in detoxification (Stern et al., 2022).
Constitutive Androstane Receptor (CAR) Agonist	A substance or ligand that binds to the CAR nuclear receptor, resulting in CAR-mediated changes in gene expression.
Constitutive Androstane Receptor (CAR) Biomarker Gene Set	A set of genes that have previously been demonstrated to be regulated by the CAR nuclear receptor and/or are modulated by substances that bind and activate CAR (Oshida et al., 2015).
Cytotoxicity	The capacity of a substance to cause damage or death to live cells.
DESeq2	A method based on the negative binomial distribution for differential analysis of count data, using shrinkage estimation for dispersions and fold changes (Love et al., 2014a, b).

Differential gene expression [differentially expressed genes] (DEGs)	Differential gene expression analysis is a set of defined statistical methods that are used to determine whether a gene is expressed differently in one condition compared to another. A differentially expressed gene (DEG) is a gene that has an increase or decrease in relative expression compared to a reference (*e.g.,* control) that meets the significance threshold defined in the differential gene expression analysis**.**
Dose-response change	A dose-response change or relationship describes the magnitude of a response in an experimental system (*e.g.,* biochemical, cell-based assay, or an organism) as a function of exposure level to a stimulus (e.g. chemical, drug, or stressor). The change correlates the amount of substance (dose) to the severity of the effect (response), generally following a curve where higher dose leads to greater effects, and this relationship can be modeled mathematically.
Dose-response modeling	A method to quantitatively assess the relationship between exposure to a chemical and the related response (effect) of that exposure.
Downregulated genes	Genes that have lower expression relative to the reference (*e.g.,* vehicle control) samples.
Enrichment analysis	A computational method for identifying pathways/gene sets or functions that are over-represented (enriched) in a list of differentially expressed genes or proteins following analysis (e.g. RNA-sequencing, proteomics), revealing coordinated biological changes and informing biological complexity beyond individual genes. This method involves statistically querying whether known gene sets from curated databases appear more frequently in the list than would be anticipated by random chance.
EPA Transcriptomic Assessment Product (ETAP)	A human health assessment approach intended for data-poor chemicals that measures changes in gene activity in short-term, animal-based studies to identify a minimum amount of chemical needed to alter gene activity and related biological processes (Mutlu et al., 2025; Brennan et al., 2024; U.S. EPA, 2024b).
False-discovery rate	A statistical method to identify and control the expected proportion of Type I errors (false positives) among all rejected null hypotheses in null hypothesis testing.
Gene Set/Pathway analysis	A bioinformatics method that interprets large biological datasets (*e.g.,* gene expression) by mapping the genes onto known, curated biological pathways/gene sets to identify meaningful patterns, reveal modes of action, and provide biological context to the list of altered data.
Gene Ontology (GO) Biological Process (BP) class [or pathway]; Gene Ontology (GO) pathway	The Gene Ontology (https://geneontology.org/) (GO) is a consortium of annotation data, ontologies, and tools to build gene ontology, assign ontology to gene/gene products, and develop software and databases to support those goals. GO's framework categorizes biological knowledge into three domains, with Biological Process describing the events within a biological activity (*e.g.,* immune response), Molecular Function describing specific biochemical activities of a gene product (*e.g.,* kinase activity), and Cellular Component describing the location where the gene product functions (*e.g.,* nucleus).
High-throughput	Method involving the rapid processing of large amounts of samples and/or data.
Histopathological	The diagnosis and study of diseases of the tissues.

Homologous genes	Genes in different species that originate from a shared ancestral gene, indicating a common evolutionary origin, but can have similar or different functional roles.
Inflammation	A protective tissue response to injury that destroys, dilutes, or walls off both the injurious agent and the injured tissue, characterized by symptoms such as pain, heat, redness, swelling, and loss of function.
In vivo	Tests or evaluations performed within an intact, living organism such as a laboratory animal or humans.
Key Event (KE)	A measurable biological change at the molecular, cellular, or tissue level that occurs after a molecular initiating event and before an adverse outcome.
Log_2 fold change	A statistic that measures the difference in expression between two conditions (*e.g.,* exposed vs control) by calculating the base-2 logarithm or their ratio, providing a symmetrical scale that is centered around zero for simpler interpretation and mathematics, with positive values indicating upregulation and negative values indicating downregulation.
Mechanism-agnostic	A method of analyzing data that is irrespective of the underlying process, method, or system involved.
Mechanistic transcriptomic analysis	Evaluating bioactivity of chemical exposure through various gene expression and pathway/gene set analyses to determine whether concerted gene changes support the proposed MOA for *p*-dichlorobenzene and provide support for the non-cancer hazard assessment for *o*-dichlorobenzene.
Mode of action (MOA)	EPA definition: Sequence of key events and processes, starting with interaction of an agent and a cell, proceeding through operational and anatomical changes, and resulting in cancer formation. A given agent may work by more than one mode of action, both at different tumor sites as well as at the same site (https://www.epa.gov/risk/conducting-human-health-risk-assessment). WHO IPCS definition: A biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data that describes key cytological and biochemical events; events are both measurable and necessary to the observed carcinogenicity (Boobis et al., 2006).
Molecular Initiating Event (MIE)	A direct interaction between a chemical or nonchemical stressor and a molecular target (*e.g.,* receptor, enzyme, and DNA) that represents the first step in a directed cascade of dependent biological processes that lead to a defined adverse outcome.
Molecular Signature Database (MSigDB)	The Molecular Signatures Database is a large, curated collection of gene sets used to understand biological states and diseases featuring diverse collections of pathways, regulatory targets, and hallmark gene sets (Castanza et al., 2023; Subramanian et al., 2005).
Necrosis	Death of tissue, usually as individual cells, group of cells, or in localized areas. (distinguished from the term “apoptosis”, which is a highly-regulated process of programmed cell death)

Nominal dose concentration	Planned exposure concentration that was administered to the test animals. Notably, nominal concentration is a different term from measured concentration (*e.g.,* within blood or tissues).
Non-genotoxic	Chemicals or substances that cause effects without directly damaging DNA or chromosomes, acting through indirect mechanisms (*e.g.,* cell proliferation, inflammation, etc.).
Orthology mapping [ortholog, orthologous]	The computational process of identifying corresponding (orthologous) genes between different species that descended from a common ancestor via speciation. Orthologs often retain similar functions across species, which can allow functional inferences (*e.g.,* rat vs. mouse).
Oxidative stress	A disturbance in the prooxidant-antioxidant balance in favor of the former, leading to cellular damage. Indicators of oxidative stress include damaged DNA bases, protein oxidation products, and lipid peroxidation products. The damage to biological tissues is caused by superoxide and other free radicals generated by many extrinsic and intracellular factors.
Point of departure (POD)	The dose-response point that marks the beginning of a low-dose extrapolation. In this report, the point is the lower bound on dose for an estimated incidence or a change in response level (*e.g.,* 1% to 10% incidence of effect) from a dose-response model (BMD).
Potency	An expression of the activity of a toxicant in terms of the concentration or amount of the toxicant required to produce a defined effect.
Principal Component Analysis (PCA)	A statistical dimensionality reduction technique used to transform high-dimensional data into a smaller set of new variables that retain most of the original information and can help with data analysis and visualization.
Probe count	The number of times a specific ligated probe sequence is detected (sequenced reads) in a sample.
P-value	A probability between 0 and 1 to indicate the likelihood of test results at least as extreme as the observed result under the assumption that the null hypothesis is correct. A small P-value would indicate that the observed outcome would be highly unlikely under the null hypothesis.
Repeat dose testing	Characterize the toxicological effects of a chemical following repeated administration for a specified period.
Sequence alignment	A computational process of arranging short RNA sequences (reads) from RNA-sequencing to a reference genome or transcriptome to identify similarities, map expression, or detect variations in sequence.
Superset	An aggregated gene list defined by all MSigDB pathway names or descriptions that include the “superset” term supporting an apical effect (*e.g.,* “apoptosis”, “necrosis”, etc.).
Transcriptomics	Study of gene expression at the RNA level.
Transcriptional [or gene expression] changes	Change of gene expression at the RNA level.

Transcriptomic point of departure (tPOD)	The experimentally derived point of departure value from dose-response modeling of transcriptomic data. There are many ways to determine a tPOD; in this report, the tPOD is determined in multiple ways, including using the lower confidence limit (BMDL) of the lowest median BMD value across at least three genes associated with a GO biological process across all tested tissues (U.S. EPA, 2024b), the lowest BMDL median of MSigDB pathways/gene sets meeting specific inclusion criteria, or the 5th percentile BMDL median of MSigDB pathways/gene sets meeting specific inclusion criteria.
Templated Oligo with Sequencing Readout (TempO-Seq)	A molecular profiling platform developed by BioSpyder Technologies, Inc. that allows for targeted gene expression analysis, designed to monitor hundreds to thousands of genes at once in high throughput format (Yeakley et al., 2017).
Toxicokinetics	The determination and quantification of the time course of absorption, distribution, metabolism, and excretion of chemicals.
Upregulated genes	Genes that have higher expression relative to the reference (*e.g.,* vehicle control) samples.

*The source of definitions for terms that may not be regarded as common knowledge in the field of toxicogenomics are indicated by the parenthetical reference where available.

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Docket
Supporting information can be found in the public docket, Docket ID EPA-HQ-OPPT-2018-0446.

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SUMMARY

EPA developed this draft technical support document (TSD) in support of the Toxic Substances Control Act (TSCA) *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene* (U.S. EPA, 2026a) and the TSCA *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene* (U.S. EPA, 2026b). The *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene* (U.S. EPA, 2026a) and *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene* (U.S. EPA, 2026b) will summarize the results of this draft TSD to evaluate the human health hazards associated with exposure to *o*-dichlorobenzene and *p*-dichlorobenzene and derive the points of departure (PODs) used to estimate risks.

TSCA was amended by the Frank R. Lautenberg Chemical Safety for the 21st Century Act on June 22, 2016. TSCA section 4(h)(1)(B) requires EPA to promote the development of alternative test methods that reduce and/or replace vertebrate animals provided the information is "of equivalent or better scientific quality and relevance for assessing risks of injury to health or the environment…" [TSCA section 4(h)(1)(B) 15 U.S.C. § 2603(h)(1)(B)]. Consistent with this mandate, on January 22, 2026, EPA recommitted to phasing out mammalian animal testing and to further incorporating New Approach Methods (NAMs) into chemical risk evaluations.[1] This draft TSD advances this commitment by providing mechanistic, human-relevant evidence, while advancing a rigorous, science-based transition away from traditional long-term animal testing using a more rapid alternative method that limits the use of animals. These shorter duration studies are a necessary bridge to validating approaches that can be used to phase out and replace traditional (*i.e.,* chronic) animal studies.

Transcriptomic-based approaches have been proposed as an alternative method to help fill data gaps for human health assessments of chemicals by providing key data on potential molecular initiating events (MIEs), mode of action (MOA), and point of departure (POD) estimates. Within the context of chemical risk assessment, transcriptomics is an experimental method that measures comprehensive changes in gene expression in response to chemical exposure and may provide a powerful line of evidence for relating exposure to possible adverse health effects.

In 2023, EPA's Office of Pollution Prevention and Toxics (OPPT) requested the National Institute of Environmental Health Sciences (NIEHS) conduct 5-day, female mouse and rat, inhalation studies with targeted transcriptomics for *o*-dichlorobenzene and *p*-dichlorobenzene for use in forthcoming TSCA draft risk evaluations (NIEHS, 2025a, b). Since that time, EPA and NIEHS have worked collaboratively to develop these data. This draft TSD describes EPA's proposed approach for analyzing and interpreting these transcriptomic data in the *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene* (U.S. EPA, 2026a) and *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene* (U.S. EPA, 2026b). To contextualize and provide additional support for the proposed approach, EPA analyzed the transcriptomic data following the prescribed *Standard Methods for Development of EPA Transcriptomic Assessment Products* (U.S. EPA, 2024b), which is summarized in Appendix B.

Gene and gene set expression were analyzed using multiple approaches that were informed by plausible mechanisms of toxicity for chemical potency analyses used in the *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene* (U.S. EPA, 2026a) and *Draft Human Health*

[1] Administrator Zeldin Gets EPA Back on Track to Eliminate Animal Testing After Biden Admin Halted Phase Out, https://www.epa.gov/newsreleases/administrator-zeldin-gets-epa-back-track-eliminate-animal-testing-after-biden-admin; accessed March 11, 2026.

and Environmental Hazard Assessment for p-Dichlorobenzene (U.S. EPA, 2026b). For *o*-dichlorobenzene, which had incomplete laboratory animal data, EPA developed a novel approach that used existing mechanistic data to inform identification of PODs specific to early biological response processes from transcriptomic data aggregated as gene sets. The Agency has proposed the use of these gene sets for transcriptomic-based POD derivation for *o*-dichlorobenzene as they are often defined from experimental or computational studies establishing functional effects at the cellular level or in some cases, as with *in vivo* genetic knockouts, organism level. Furthermore, custom-curated lists of these gene sets can be identified that provide a means to anchor changes in these gene sets with specific phenotypes/mechanisms of interest based on the available hazard database. These gene set-based PODs were compared with POD values from traditional (*i.e.,* chronic) toxicity studies in the same organs to improve confidence in the values used for risk estimates and to make use of the best available science.

The transcriptomic point of departure (tPOD) value, based on the 5th percentile of the distribution of these gene sets for liver and lung effects, was ultimately selected as a representative marker for the level of *o*-dichlorobenzene that could cause short-term liver and lung harm. These transcriptomic-supported PODs are being used as co-critical PODs in the evaluation of hazard for *o*-dichlorobenzene inhalation exposure, oral exposure, and skin contact (U.S. EPA, 2026a). EPA determined that the 5th percentile of the distribution of the statistical lower confidence limit on the benchmark dose (*i.e.,* benchmark dose lower bound) estimates of these gene sets is an appropriate value to use as the tPOD as this generally aligned with more sensitive (but not overly sensitive) gene set changes that occur just prior to the inflection point leading to maximal gene expression activation. Additionally, the 5th percentile distribution-based approach aligns with established EPA risk assessment approaches in other regulatory contexts, such as for deriving numerical national water quality criteria (U.S. EPA, 1985), the Threshold of Toxicological Concern (Kroes et al., 2005), and species sensitivity distribution analysis (Dhond and Barron, 2022; Newman et al., 2000). Furthermore, within i*n vitro* NAMs, the lower 5th percentile of the distribution of benchmark dose values has been used to identify PODs based on preliminary chemical bioactivity screening (Friedman et al., 2020) and in the field of toxicogenomic research using the distribution of benchmark doses of gene sets in multiple *in vitro* high-throughput transcriptomic analyses (Bundy et al., 2024; Harrill et al., 2024; Harrill et al., 2021).

This tPOD approach that utilizes the 5th percentile derived across a distribution of gene sets differs from other short-term *in vivo* transcriptomic methods that present a tPOD based on either the single most sensitive gene set or the 5th percentile of genes within a single set (U.S. EPA, 2024b; NTP, 2018). The 5th percentile of the distribution of the benchmark dose lower bound approach is discussed in more detail in Section 4.2.3 and is visually represented in Figure 4-12, Figure 4-14, Figure 4-16, Figure 4-18, Figure 4-20, and Figure 4-22.

For *p*-dichlorobenzene, chronic studies in rodents demonstrate that exposure causes liver tumors in mice but not in rats, and this hepatic tumorigenesis is proposed to be caused by activation of the constitutive androstane receptor (CAR). Furthermore, CAR-mediated hepatic liver tumors in rodents have been determined by regulatory agencies as not relevant to human liver tumorigenesis. EPA found that key genes responsive to CAR activity were disrupted after exposure in mice and to a lesser degree in rats, potentially accounting for the observed species-specific differences in liver tumor outcomes. This finding provided evidence of a plausible molecular initiating event (MIE) linking *p*-dichlorobenzene exposure with an established species-specific MOA for hepatocellular proliferation and liver tumorigenesis in mice as summarized in the *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene* (U.S. EPA, 2026b). Integrating the results of this analysis with the conclusions described in the *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene* (U.S. EPA, 2026b), which followed the EPA *Guidelines for Carcinogen Risk*

Assessment (U.S. EPA, 2005), these data support the conclusion that *p*-dichlorobenzene is "*Not Likely to be Carcinogenic to Humans*."

This draft TSD is being released for public comment and peer review by the independent Science Advisory Committee on Chemicals (SACC) in June 2026. EPA is soliciting input on topics associated with experimental design, analysis methodology, soundness of conclusions, and use of the results to support the *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene* (U.S. EPA, 2026a) and *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene* (U.S. EPA, 2026b).

1 INTRODUCTION

The mission of EPA is to protect human health and the environment. The Agency routinely performs human health risk assessments to estimate the nature and probability of adverse health effects in humans exposed to chemicals. These assessments typically rely on traditional animal bioassays to observe potential adverse health effects from chemical exposure and to identify PODs[2] used in the derivation of health reference values. Although these traditional toxicity studies have a long history of use in risk characterization and risk management, their application is limited due to substantial costs, lengthy test durations, and large numbers of test animals. This has resulted in a large disparity between the vast numbers of chemicals that humans are exposed to and the small number of well-studied chemicals with animal or human data. Furthermore, these studies still have uncertainties that often do not answer the most fundamental needs of a risk assessment, namely how chemical exposures elicit physiological responses in humans, referred to as the mode of action (MOA).[3] Many world-wide health organizations, including EPA, have been developing alternative approaches with and without whole animal testing for many years (NRC, 2007). Transcriptomic-based approaches have been proposed as an alternative method to help fill data gaps for human health hazard assessments of chemicals by providing comprehensive characterization of potential MIEs, MOA, and POD estimates. One alternative approach to reducing animal use involves employing transcriptomics from short-term studies to inform MOA and POD determination, thereby reducing reliance on animal toxicity studies that necessitate higher numbers of animals and longer exposure durations.

1.1 Overview of Transcriptomics

Comprising both coding and non-coding regions of the genome, deoxyribonucleic acid (DNA) serves as a molecular blueprint which is transcribed into a ribonucleic acid messenger (mRNA) and ultimately translated into proteins that provide function to living cells. Transcriptomics refers to a high-throughput method of comprehensively assessing the expression of some or all genes across an organism's genome at a specific moment in time. In chemical risk assessment, this typically involves measuring relative changes in mRNA-transcripts after exposure to a chemical compared with a control. Transcriptomic data analysis allows characterization of the biological processes or gene sets that are perturbed after exposure to a chemical. Transcriptomic data provides a sensitive measure of chemical-induced bioactivity since changes in gene expression are commonly observed to precede or coincide with apical effects (Hester S, 2016; Thomas et al., 2012a; Thomas et al., 2011; Lobenhofer et al., 2004b). Moreover, the use of transcriptomic data for chemical risk assessment has been proposed previously by EPA and standardized methods and software applications have been developed for quantifying dose-responsive transcriptomic changes using methods traditionally applied in chemical risk assessment, providing insight into chemical potency for biological perturbations (U.S. EPA, 2024b; NTP, 2018; U.S. EPA, 2009; Yang et al., 2007).

[2] In human health risk assessment practice, a point of departure (POD) represents the dose-response point that marks the beginning of a low-dose extrapolation. This point can be the lower bound on dose for an estimated incidence or a change in response level from a dose-response model (*e.g.*, benchmark dose; BMD), or a no-observed-adverse-effect level (NOAEL) or lowest-observed-adverse-effect level (LOAEL) for an observed incidence or change in level of response. For BMDs, this is typically the BMD lower confidence bound (BMDL).

[3] A MOA describes a chemical-specific sequence of key biological events that leads to an adverse health outcome, integrating mechanistic and apical data to support hazard identification and risk assessment (Boobis et al., 2006). In contrast, an adverse outcome pathway (AOP) is a chemical agnostic framework that organizes existing knowledge by linking a molecular initiating event (MIE) to an adverse outcome through a series of Key Events (KEs) along increasing levels of biological organization, without implying causation for any specific substance (Ankley et al., 2010). Although AOPs do not establish chemical-specific mechanisms, they provide an organized framework for evaluating whether a chemical's toxicological profile aligns with an established AOP.

A transcriptomic point of departure (tPOD) is the experimentally derived POD value from dose-response modeling of transcriptomic data. Transcriptomic dose-response modeling is performed to determine benchmark doses (BMDs); BMDs are defined as the dose or concentration that produces a specific benchmark response (BMR), which is the individual gene(s) or aggregated gene sets that produce an effect. Structured models of gene sets are also described as pathways, and the term "pathways" is frequently used throughout the literature to describe both gene sets and pathways; it is more precise to use the term "gene sets" for curated lists of genes, and "pathways" for structure models of gene sets. Multiple studies, as short as five days in duration, have demonstrated high concordance between the gene set-based transcriptional BMDs (and the lower 95% confidence bound benchmark dose lower bound [BMDL]) and those derived from apical effects in traditional *in vivo* toxicity studies (see Figure 1-1 below) (Gwinn et al., 2020; Johnson et al., 2020; Zhou et al., 2017; Chepelev et al., 2015; Clewell et al., 2014; Jackson et al., 2014; Thomas et al., 2012b; Clewell et al., 2011; Lobenhofer et al., 2004a).

While previous efforts have examined short-term exposures of varied durations (U.S. EPA, 2024a), the 5-day exposure duration has been recommended by the National Toxicology Program (NTP) (NTP, 2018). As a result, transcriptomic analyses from 5-day animal exposure studies have been proposed as a means to improve the risk assessment process and enhance testing paradigms because it (1) allows for a more rapid assessment of chemicals compared to traditional (*i.e.,* chronic) toxicity testing; (2) offers increased, highly sensitive detection of gene expression changes which underpin the observable (apical) health effects; (3) reduces and refines animal usage through shorter duration assays; (4) provides characterization of critical cellular and molecular events to support the development of biologically plausible MOAs through identifying specific causal gene sets and/or pathways that may be linked with adverse apical effects; and (5) can help prioritize chemicals for further assessment or assist in identifying critical data gaps in a cost and time-effective manner.

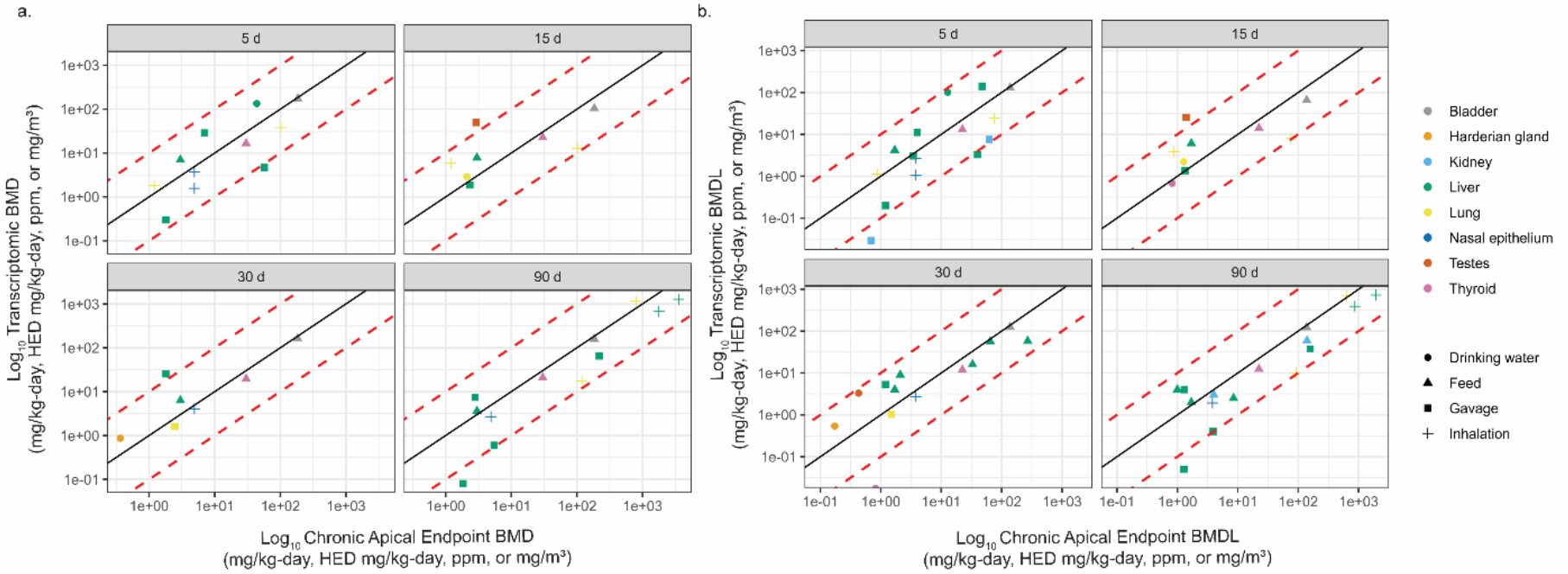


Figure 1-1. Concordance Between Mouse or Rat Transcriptomic and Chronic Apical Benchmark Doses (BMDs) or Benchmark Dose Lower Bounds (BMDLs) Including Carcinogenicity Studies (U.S. EPA, 2024a). (a.) Transcriptomic BMDs vs. Two-Year Apical BMDs. (b.) Transcriptomic BMDL Values vs. Two-Year Apical BMDL Values

Transcriptomic results from 1, 2, 3, 5, 6, or 7-day test durations are reported under the 5-day timepoint. Results from 15 and 21-day test durations are reported under the 15-day timepoint. Results from the 28, 29, 30, and 31-day exposure durations are reported under the 30-day timepoint, and results from the 90 and 91-day exposure durations are reported under the 90-day timepoint. Only studies with a transcriptomic BMD and/or BMDL and 2-year apical BMD and/or BMDL were included in the graphs. For the transcriptomic BMD and/or BMDL, the median of the most sensitive gene set or datapoint (most similar to the EPA Transcriptomic Assessment Product approach) was used. If multiple tissues were investigated in the study, then the lowest transcriptomic BMD(L) across all tissues was selected. For the apical BMD(L), the lowest 2-year BMD(L) reported for a chemical and species was used, which sometimes did not match the tissue used for obtaining the transcriptomic BMD(L). If the same chemical was independently tested in different studies, then the transcriptomic BMD(L) and associated apical BMD(L) for each study was included. Only dose-matched metrics were used. For instance, if the apical BMD(L) was in mg/kg-day, then the transcriptomic BMD(L) reported in mg/kg-day was used, but if the apical BMD(L) was in ppm, then the transcriptomic BMD(L) reported in ppm was used. The header for each facet of the figure indicates the timepoint in days (d) for the transcriptomic BMD(L). HED is human equivalent dose. The solid line indicates a perfect concordance at 1. The dashed lines represent 10-fold difference between the BMD(L) values.

1.1 5-Day Transcriptomic Studies of *o*-Dichlorobenzene and *p*-Dichlorobenzene

In 2023, EPA's OPPT requested NIEHS to conduct 5-day, *in vivo*, inhalation studies with targeted transcriptomics for *o*-dichlorobenzene and *p*-dichlorobenzene for use in forthcoming TSCA draft risk evaluations (NIEHS, 2025a, b). Since that time, EPA and NIEHS have worked collaboratively to develop these data which are summarized briefly in Section 0 and are provided publicly (https://doi.org/10.22427/NIEHS-DATA-NIEHS-13; accessed March 13, 2026). Because *p*-dichlorobenzene and *o*-dichlorobenzene have existing databases describing tissue-specific apical outcomes, a focused experiment was designed that used female mice and rats and five targeted tissues of interest (heart, kidney, liver [left lobe], lung, and ovary).

This draft TSD describes EPA's proposed approach for analyzing and interpreting these transcriptomic data in the *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene* (U.S. EPA, 2026a) and *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene* (U.S. EPA, 2026b). This draft TSD represents a significant advancement by EPA in the use of transcriptomic evaluations for MOA development in support of identifying tPODs relevant to adverse human health effects. This document provides the technical details of the transcriptomic analysis and associated methods. The full integration of these results with the apical health effect data can be found in the *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene* (U.S. EPA, 2026a) and *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene* (U.S. EPA, 2026b).

2 CHEMICAL IDENTITY

Table 2-1. Chemical Identity of 1,2-Dichlorobenzene (*o*-Dichlorobenzene)

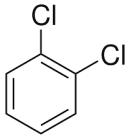
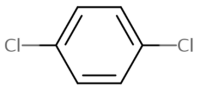
Property	Value
Chemical structure	
DTXSID	DTXSID6020430
CASRN	95-50-1
IUPAC Name	1,2-dichlorobenzene
Synonyms	ortho-dichlorobenzene *o*-dichlorobenzene Benzene, 1,2-dichloro-

Table 2-2. Chemical Identity of 1,4-Dichlorobenzene (*p*-Dichlorobenzene)

Property	Value
Chemical structure	
DTXSID	DTXSID1020431
CASRN	106-46-7
IUPAC Name	1,4-dichlorobenzene
Synonyms	para-dichlorobenzene *p*-dichlorobenzene Benzene, 1,4-dichloro- Benzene, p-dichloro-

3 ANIMAL STUDY

The complete methods and data for the 5-day, *in vivo*, inhalation studies with targeted transcriptomics were performed by the NIEHS Division of Translational Toxicology (DTT) for *o*-dichlorobenzene and *p*-dichlorobenzene, are available, and will not be described here in full detail (NIEHS, 2025a, b). Summaries of the primary study parameters can be found in Table 3-1 through Table 3-4.

Table 3-1. Summary of Study Parameters for *o*-Dichlorobenzene in Rats

Parameter	Value
Species	Rat
Strain	Hsd: Sprague Dawley (SD)
Sex	Female
Age	7–8 weeks post acclimation
Sample Size	n = 10 vehicle control; n = 5 treatment group
Route of Exposure	Whole-body Inhalation
Vehicle	Preheated nitrogen
Doses	0, 1, 10, 30, 100, 250, 500 ppm
Dosing Frequency	Once per day for 6 hours plus T_{90}
Dosing Duration	5 days
Sacrifice Time After Last Dose	24 hours
Organs Evaluated	Heart, kidney, liver, lung, and ovary

Table 3-2. Summary of Study Parameters for *p*-Dichlorobenzene in Rats

Parameter	Value
Species	Rat
Strain	Hsd: Sprague Dawley (SD)
Sex	Female
Age	8–9 weeks post acclimation
Sample Size	n = 10 vehicle control; n = 5 treatment group
Route of Exposure	Whole-body Inhalation
Vehicle	Preheated nitrogen
Doses	0, 1, 10, 50, 150, 400, 800 ppm
Dosing Frequency	Once per day for 6 hours plus T_{90}
Dosing Duration	5 days
Sacrifice Time After Last Dose	24 hours

Table 3-2. Summary of Study Parameters for *p*-Dichlorobenzene in Rats

Parameter	Value
Organs Evaluated	Heart, kidney, liver, lung, and ovary

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Table 3-3. Summary of Study Parameters for *o*-Dichlorobenzene in Mice

Parameter	Value
Species	Mouse
Strain	B6D2F1/Crl
Sex	Female
Age	9–10 weeks post acclimation
Sample Size	n = 10 vehicle control; n = 5 treatment group [a]
Route of Exposure	Whole-body Inhalation
Vehicle	Preheated nitrogen
Doses	0, 1, 10, 30, 100, 250 ppm
Dosing Frequency	Once per day for 6 hours plus T_{90}
Dosing Duration	5 days
Sacrifice Time After Last Dose	24 hours
Organs Evaluated	Heart, kidney, liver, lung, and ovary
[a] The 250 ppm dose group had a sample size of n = 8, with 1 animal euthanized moribund and excluded.	

Table 3-4. Summary of Study Parameters for *p*-Dichlorobenzene in Mice

Parameter	Value
Species	Mouse
Strain	B6D2F1/Crl
Sex	Female
Age	6–7 weeks post acclimation
Sample Size	n = 10 vehicle control; n = 5 treatment group
Route of Exposure	Whole-body Inhalation
Vehicle	Preheated nitrogen
Doses	0, 1, 10, 50, 150, 400 ppm
Dosing Frequency	Once per day for 6 hours plus T_{90}
Dosing Duration	5 days
Sacrifice Time After Last Dose	24 hours
Organs Evaluated	Heart, kidney, liver, lung, and ovary

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4 GENE EXPRESSION ANALYSES AND DERIVATION OF POINTS OF DEPARTURE FROM 5-DAY *IN VIVO* TRANSCRIPTOMIC STUDIES

As part of EPA's efforts to apply transcriptomic-based approaches for human health risk assessments, EPA explored multiple approaches for interpreting the transcriptomic data provided by the NIEHS studies within the context of the *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene (*U.S. EPA, 2026a*)* and *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene (*U.S. EPA, 2026b*)* and using the best available science:

1. As an initial approach, EPA examined changes in gene expression which can inform how chemicals cause different effects across dose levels, tissues, and animals, which is described in Section 4.1.
    a. Method: Identify differentially expressed genes (DEGs) for each chemical, species, and tissue to assess the magnitude of response to chemical exposure across dose levels.
2. EPA performed dose-response modeling on gene expression data, after which different sources and aggregations of publicly available gene sets were used to curate more biologically relevant terms than what gene-level analyses can offer. These analyses are described in Section 4.2.
    a. Method: Derive the lowest Gene Ontology Biological Process (GOBP) tPOD following the *Standard Methods for Development of EPA Transcriptomic Assessment Products* (U.S. EPA, 2024b), which uses the BMDL of the lowest BMD median from the GOBP gene set database to identify a tPOD. Complete results for this analysis are shown in Appendix B.
    b. Method: Derive the 5th percentile GOBP tPOD using the median BMDL gene set distribution from the GOBP gene set with at least three genes. Complete results are shown in Section 4.2.3(*o*-dichlorobenzene) and Appendix C (*p*-dichlorobenzene).
    c. Method: Derive the lowest Molecular Signature Database (MSigDB) tPOD considering the full landscape of MSigDB gene sets. Complete results are shown in Section 4.2.3 (*o*-dichlorobenzene) and Appendix C (*p*-dichlorobenzene).
    d. Method: Derive mechanism-informed tPODs using the MSigDB gene set BMDL Median based on either the lowest or 5th percentile value for pathways involving potential key events across an MOA. Complete results are shown in Section 4.2.3 (*o*-dichlorobenzene) and Appendix C (*p*-dichlorobenzene).
3. EPA identified if short-term exposure to *p*-dichlorobenzene or *o*-dichlorobenzene results in Constitutive Androstane Receptor (CAR) activation, which is the proposed MIE for *p*-dichlorobenzene-induced liver tumors in mice. This analysis is described in Section 4.3 for *p*-dichlorobenzene and Appendix D for *o*-dichlorobenzene.
    a. Method: Apply a biomarker analysis to investigate the potential role of CAR in *p*-dichlorobenzene and *o*-dichlorobenzene hazard evaluations.

4.1 Qualitative Differential Gene Expression Analysis

This section describes EPA's initial approach to quantify changes in gene expression in the 5-day transcriptomic studies to inform how *o*-dichlorobenzene and *p*-dichlorobenzene cause different effects across dose levels, tissues, and animals. As part of an exploratory, qualitative analysis, DEGs were identified for each chemical (*o*-dichlorobenzene and *p*-dichlorobenzene), species (mouse and rat) and tissue (liver, lung, kidney, heart, and ovary) by contrasting each dose level relative to the vehicle control

using DESeq2[4] (Love et al., 2014a). From this analysis, liver was identified as one of the most responsive tissues across both species and chemicals as it typically produced more DEGs than other tissues at matched dose levels (Figure 4-1; Figure 4-2). For *o*-dichlorobenzene, the tissues with the second- and third-most DEGs in mouse and rat were lung and kidney, respectively, whereas for *p*-dichlorobenzene, the tissues with the second- and third-most DEGs in both species were heart and ovary. Notably, the number of DEGs identified for either chemical was higher at the highest dose tested than all lower dose levels. As noted in Appendix A.2 (Table_Apx A-2), multiple samples of mouse kidney had evidence of tissue contamination and were excluded from analysis resulting in lower sample sizes for kidney compared to the other tissues. However, all remaining kidney samples across the vehicle controls and dose groups for both *o*-dichlorobenzene and *p*-dichlorobenzene were within the minimum sample size recommendation for DESeq2 analysis (Li et al., 2022).

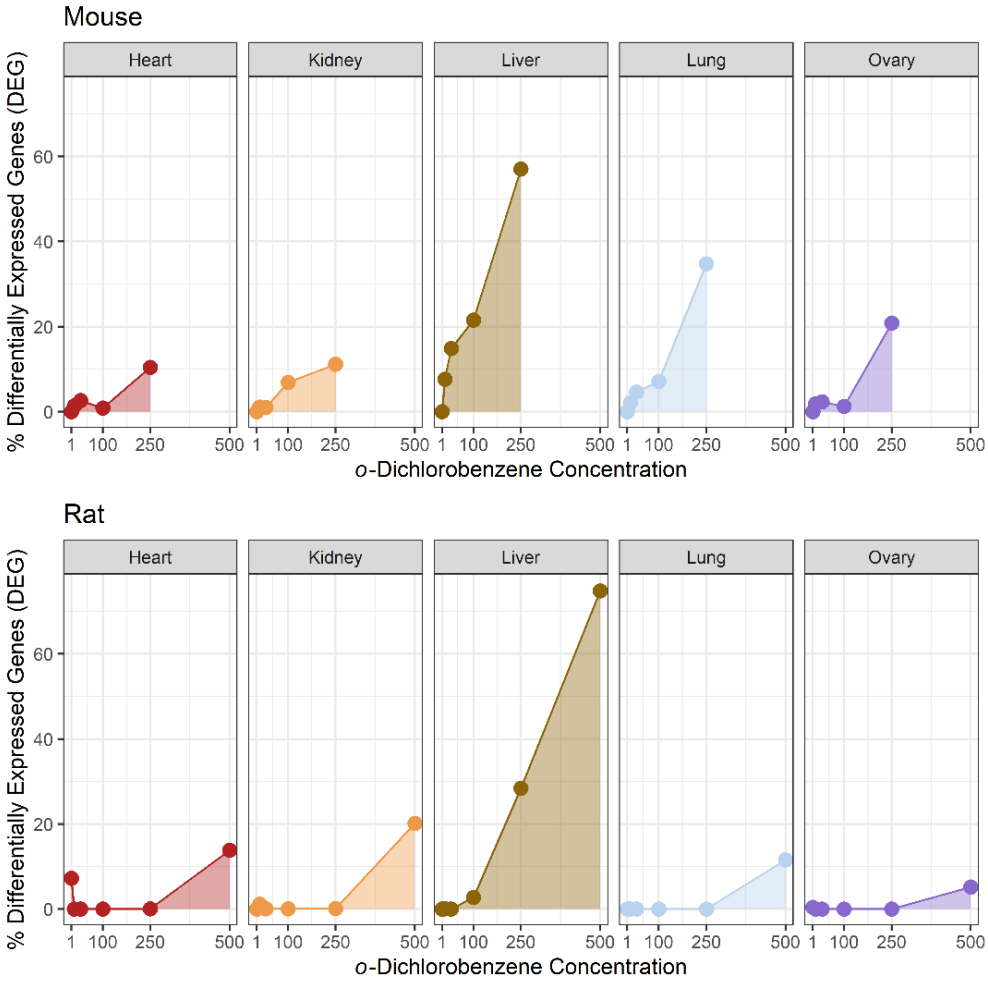

Figure 4-1. Differentially Expressed Gene (DEG) Plot Showing the Percent of Analyzed Genes That Are Differentially Expressed

Note: adjusted p-value ≤0.05 and absolute $\log_2$ fold change ≥$\log_2$ (1.5) following Wald test using DESeq2) at each concentration relative to control at each concentration following exposure to *o*-dichlorobenzene by tissue shows that liver has the greatest magnitude of differential expression in both rat and mouse.

[4] DESeq2 is a publicly available software package in the R statistical programming language and is used to analyze RNA-sequencing studies to identify differentially expressed genes between different experimental conditions. This software package uses statistical methods based on the negative binomial distribution for differential analysis of count data, provides data normalization methods and shrinkage estimation, and is a valuable tool for research in gene expression.

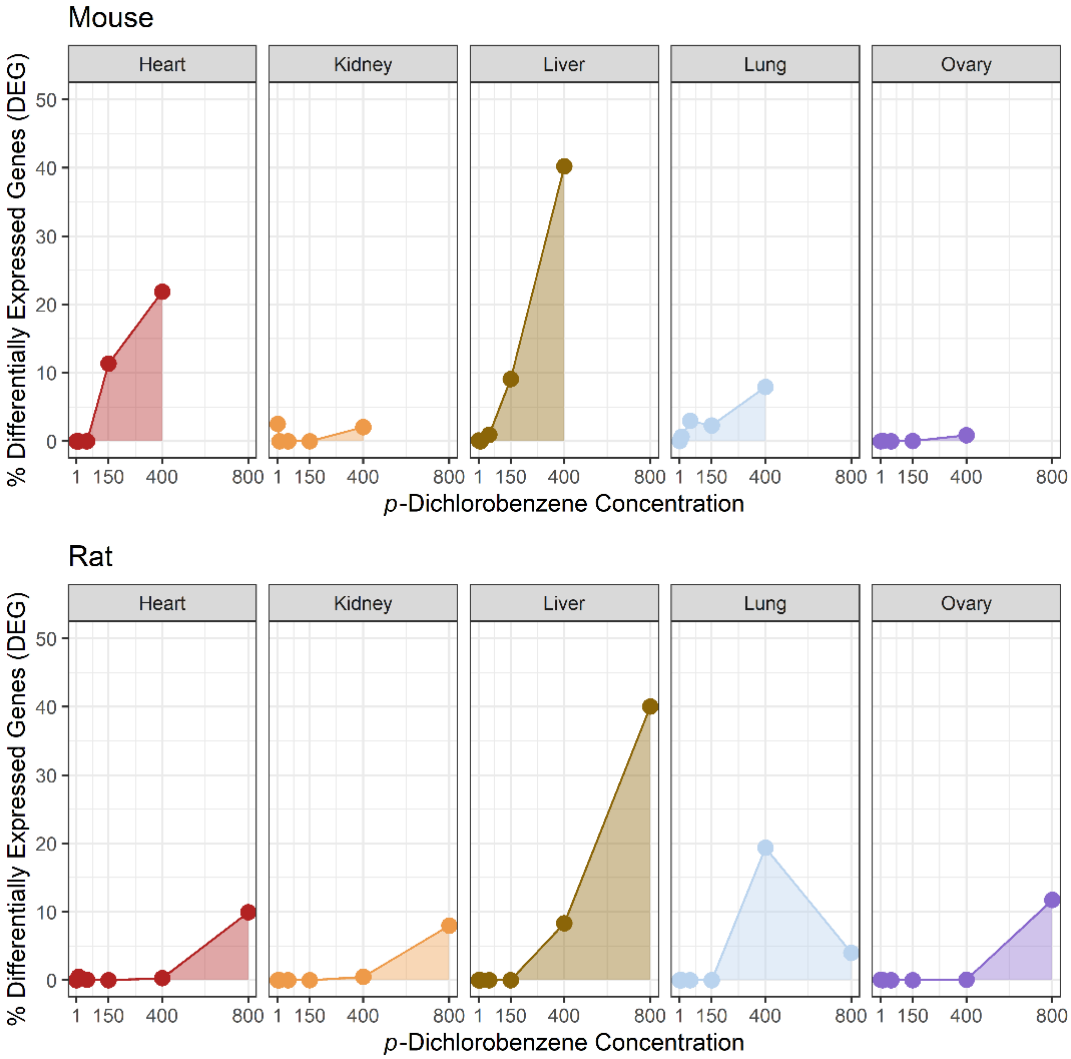

Figure 4-2. Differentially Expressed Gene (DEG) Plot Showing the Percent of Analyzed Genes That Are Differentially Expressed

Note: adjusted p-value ≤0.05 and absolute $\log_2$ fold change ≥$\log_2(1.5)$ following Wald test using DESeq2) at each concentration relative to control following exposure to *p*-dichlorobenzene by tissue shows that liver has the greatest magnitude of differential expression in both rat and mouse.

4.2 Quantitative Transcriptomic Analysis of Biological Gene Sets to Derive Points of Departure

This section provides an overview of the dose-response modeling that EPA performed on the 5-day *in vivo* transcriptomic data. The Agency interpreted gene expression responses multiple ways by using different sources and aggregations of publicly available gene sets which curate more biologically relevant terms than what gene-level analyses can offer. Using these curated gene sets derived from experimental and computational methods, as well as literature-based sources (The Gene Ontology Consortium, 2025; Castanza et al., 2023; Liberzon et al., 2015; Subramanian et al., 2005; Ashburner et al., 2000), EPA then applied several methods to estimate tPODs for liver, lung, and kidney tissues in both mice and rats for *o*-dichlorobenzene. EPA performed the same analysis described here, along with additional analyses, to estimate tPODs for *p*-dichlorobenzene; these analyses can be found in Appendix C Because a review of existing toxicity data for *o*-dichlorobenzene identified adverse health outcomes in

liver, lung, and kidney (Mörbt et al., 2011; ATSDR, 2006; U.S. EPA, 2006; Zissu, 1995; Hayes et al., 1985; NTP, 1985; Haskell Laboratories, 1982; Hollingsworth et al., 1958), only analyses in those tissues are presented.

4.2.1 Selection of Aggregated Gene Supersets

Grouping individual genes into predefined collections, or gene sets, based on specific biological pathways, processes, or molecular functions is a common method used to interpret and provide biological context to the observed changes in transcriptomic studies (Subramanian et al., 2005). While enrichment of these gene sets does not necessarily result in an adverse apical outcome, changes in these gene sets are often experimentally and computationally linked to cellular-level responses that, when combined with mechanistic data, can relate to and inform apical effects. Additionally, several studies have shown high concordance between apical effect BMDs from traditional toxicity studies and BMDs derived from gene sets from short-term *in vivo* transcriptomic studies [reviewed in (U.S. EPA, 2024a)]. To this end, EPA selected two gene set databases to aggregate and summarize the gene-level dose-response modeling results.

The first gene set database is GOBP used in the Gene Ontology category analysis module within BMDExpress 2.3 that follows the *Standard Methods for Development of EPA Transcriptomic Assessment Products* (U.S. EPA, 2024b; Phillips et al., 2019); GOBP gene sets are derived directly from Gene Ontology (GO) terms developed by the expert-driven Gene Ontology Consortium as a structured, controlled vocabulary to link biological data across species using three functional categories based on hierarchical relationships (The Gene Ontology Consortium, 2025; Ashburner et al., 2000). The second is all of the annotated gene sets from MSigDB, which consists of gene sets that are reviewed, curated, and annotated manually (Castanza et al., 2023; Liberzon et al., 2015; Subramanian et al., 2005). MSigDB gene sets are inclusive of Hallmark, human chromosome cytogenic band positional gene sets, curated gene sets (including canonical pathways, BioCarta, WikiPathways, KEGG, and Reactome), regulatory target gene sets (including those related to microRNAs and transcription factors), computational gene sets (including those linked to cancer), Gene Ontology gene sets, oncogenic gene sets, and immunologic gene sets (Castanza et al., 2023; Liberzon et al., 2015; Subramanian et al., 2005).

The MSigDB has coverage over an extremely diverse landscape of biological mechanisms with 50,236 total unique accession numbers for gene sets in the version used for this analysis. This broad representation of potential toxicities creates a valuable background for untargeted examination of potential chemical-induced transcriptomic effects. The MSigDB gene sets for mouse were mouse-native gene sets (meaning that these databases were curated specifically for pathways relating to mouse-specific cell and molecular functions), whereas rat-specific gene sets required additional functional orthology mapping to the human gene set equivalents because there are no rat native gene sets available in the MSigDB. Orthology mapping was performed using an NCBI orthology mapping package, Orthology.eg.db (DOI: 10.18129/B9.bioc.Orthology.eg.db) and genome-wide annotations for mouse, rat, and human (DOI: 10.18129/B9.bioc.org.Mm.eg.db). The MSigDB collection of gene sets was further categorized into groups of expertly curated terms which are described in more detail below.

To investigate dose-responsive changes in gene expression associated with potential mechanistic key events (KEs) for *o*-dichlorobenzene, including oxidative stress and cell death (Mörbt et al., 2011; ATSDR, 2006; U.S. EPA, 2006; Zissu, 1995; Hayes et al., 1985; NTP, 1985; Haskell Laboratories, 1982; Hollingsworth et al., 1958), heat maps were visualized across the full landscape of genes within MSigDB gene sets with at least three genes categorized into the following “supersets”: apoptosis, cytotoxicity, inflammation, oxidative stress, necrosis, or a combination of all five terms. It is important to note that there is varying overlap between genes within each of these five supersets, owing to the

functional connections and overlap observed across many of these discrete cellular processes. UpSet plots, a data visualization method for showing the overlap between multiple sets, for rat (Figure 4-3) and mouse (Figure 4-4) show that the apoptosis-related MSigDB gene sets have the greatest unique representation. This means that genes related to apoptosis comprised the largest proportion of unique DEGs that were not shared with any other superset space. The overlap between genes in aggregated gene sets is always present in enrichment analyses; therefore, the aggregated supersets using the combination of genes within any of the five terms relating to the plausible MOA presented may be most appropriate to gather a sense of the large-scale biological impacts of exposure. For rats, there were 1,095 total unique genes annotated to the 5 supersets identified as relevant for the plausible MOA described. Of those, 236 were unique to apoptosis, 122 to inflammation, 43 to cytotoxicity, 35 to oxidative stress, and 3 to necrosis. For mice, there were 745 total unique genes annotated to the 5 relevant supersets. Of those, 281 were unique to apoptosis, 60 to inflammation, 43 to oxidative stress, 9 to necrosis, and 5 to cytotoxicity.

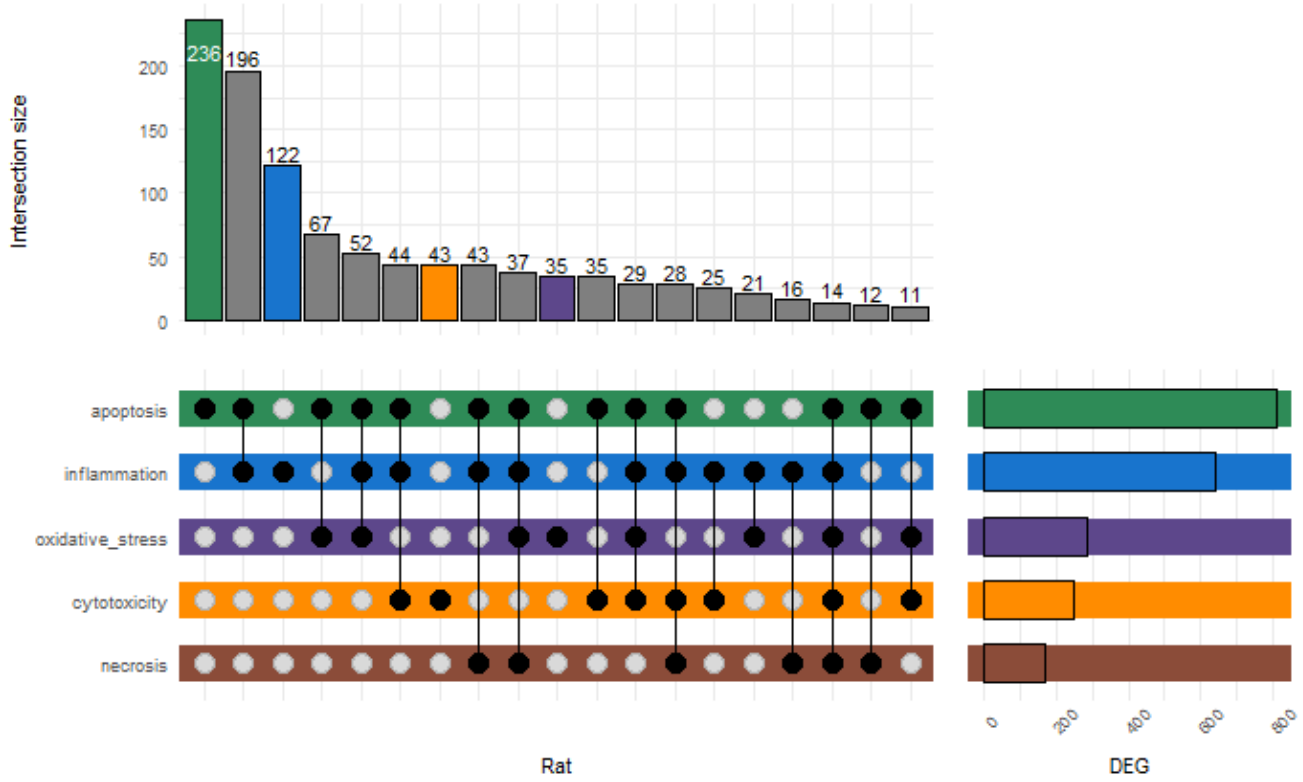

Figure 4-3. UpSet Plot Showing the Overlap of Mouse Genes Included as Inputs in BMD Modeling
Mouse genes mapped to each of the MSigDB gene set terms in a superset using the five terms determined from the *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene* (U.S. EPA, 2026a): "Apoptosis," "Cytotox," "Inflam,: "Oxidative," and "Necrosis." The UpSet plot shows "sets" of DEGs in the bottom-right corner, highlighting the total number of DEGs involved in each superset. The "intersections" of sets in the bar graph along the top show the 'exclusive intersection', which represents the total number of unique DEGs attributable to each superset or intersection of supersets. The black circles highlight which sets are included in each possible intersection across supersets. Only intersections with at least 10 members are shown.

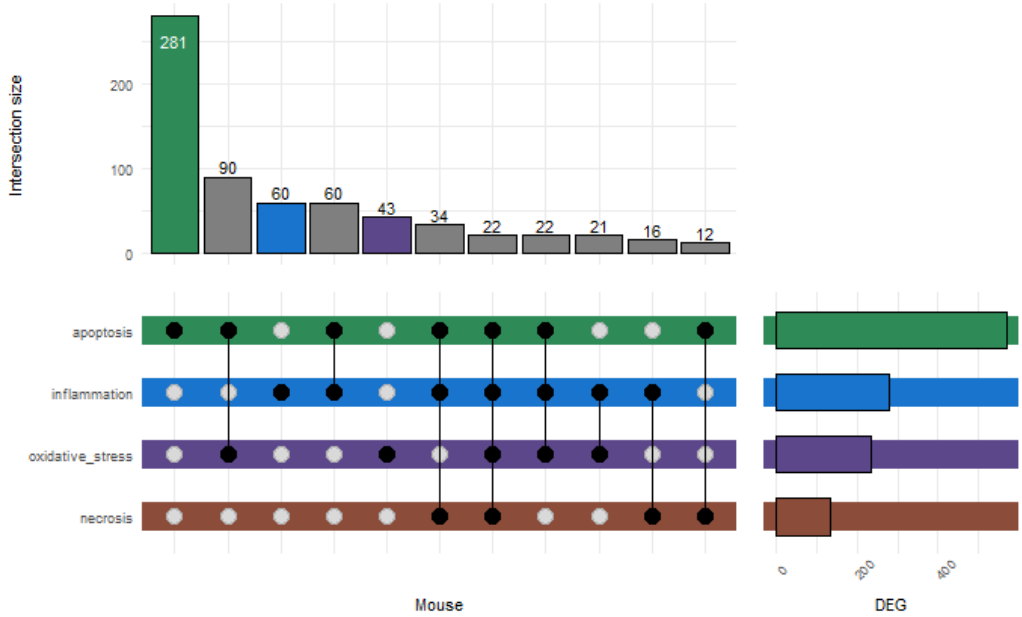

Figure 4-4. UpSet Plot Showing the Overlap of Mouse Genes Included as Inputs in BMD Modeling
Mouse genes mapped to each of the msigdb gene set terms in a superset using the five terms determined from the *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene* (U.S. EPA, 2026a): "Apoptosis," "Cytotox," "Inflam," "Oxidative," and "Necrosis." The UpSet plot shows "sets" of DEGs in the bottom-right corner, highlighting the total number of DEGs involved in each superset. The "intersections" of sets in the bar graph along the top show the 'exclusive intersection', which represents the total number of unique DEGs attributable to each superset or intersection of supersets. The black circles highlight which sets are included in each possible intersection across supersets. Only intersections with at least 10 members are shown; cytotoxicity included only 5 unique members and did not have any intersections with at least 10 members; therefore, it is not visualized in this graphic.

4.2.2 Superset Expression Analysis for *o*-Dichlorobenzene

Visualizations of the concentration-responsive gene expression changes after *o*-dichlorobenzene exposure within each tissue were performed using heatmaps (Figure 4-5 through Figure 4-10). These heatmaps display the dose-level summary $\log_2$ fold change of each gene within each of the five MSigDB supersets compared to control as a row, with upregulated genes in green and downregulated genes in purple. These results demonstrate the concentration-responsive nature of the genes within each superset and highlight the magnitude of changes, especially within the liver and lung. Differential expression of the MSigDB gene sets of interest occurred primarily at the highest test concentrations. Although this pattern of enrichment confirms that the dosing regimen encompassed a biologically-relevant exposure range, the steep slope of some of these responses would limit the number of genes amenable to concentration-response modeling.

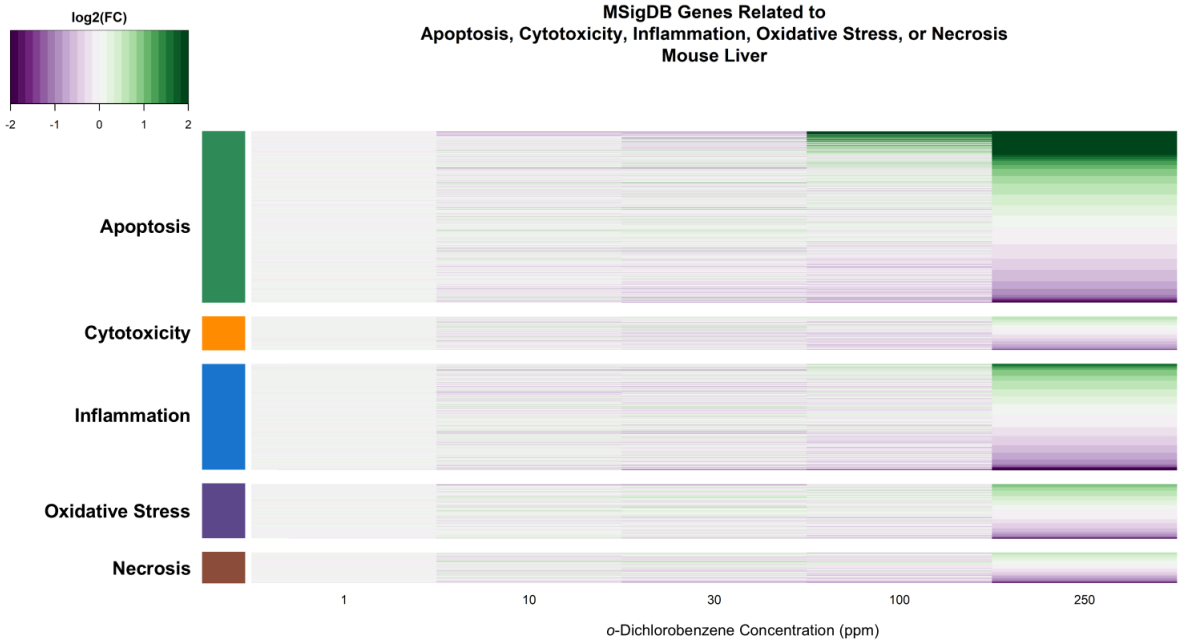

Figure 4-5. Heatmap of the Mouse Liver Gene Set Deregulation (Represented as Log_2 Fold Change) Following *o*-Dichlorobenzene Exposure of Each Gene Annotated to the Five MSigDB Supersets

Upregulated genes are displayed in green and downregulated genes in purple with color density indicating the relative strength of the change**.**

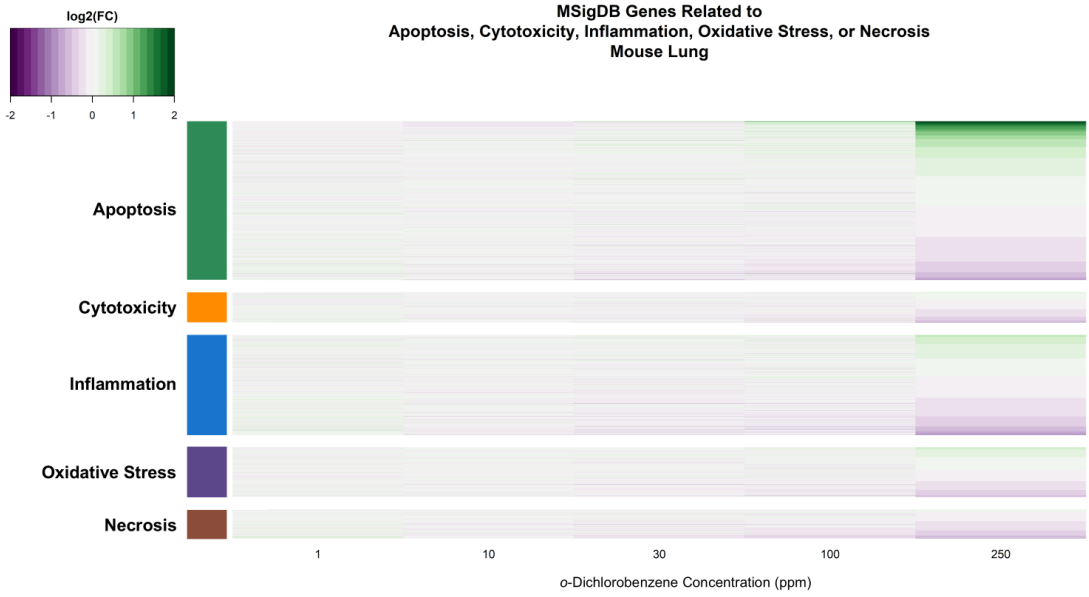

Figure 4-6. Heatmap of the Mouse Lung Gene Set Deregulation (Represented as Log_2 Fold Change) Following *o*-Dichlorobenzene Exposure of Each Gene Annotated to the Five MSigDB Supersets

Upregulated genes are displayed in green and downregulated genes in purple with color density indicating the relative strength of the change.

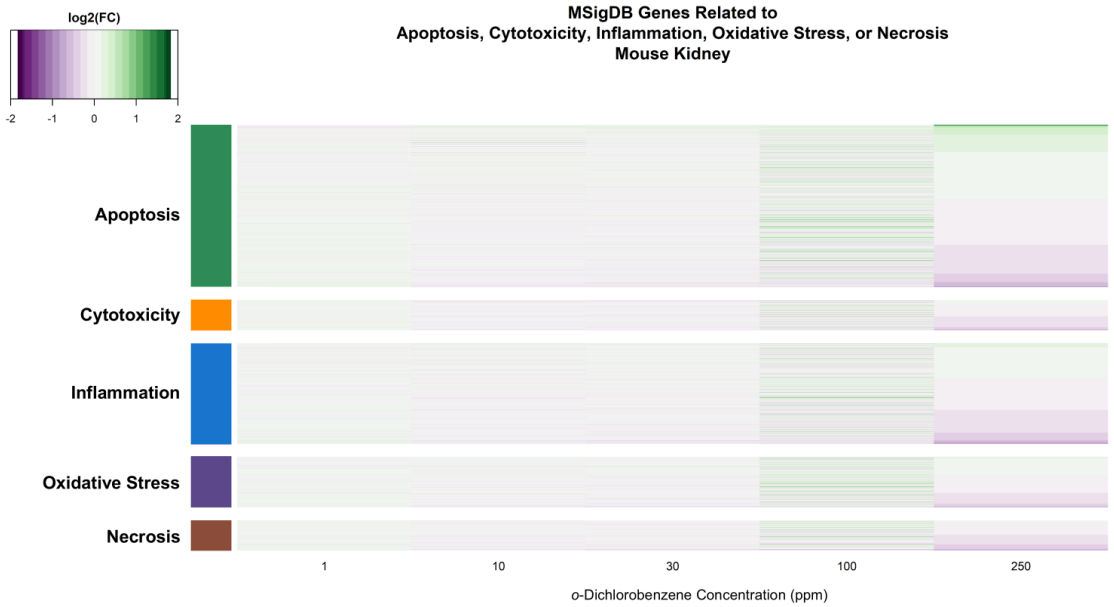

Figure 4-7. Heatmap of the Mouse Kidney Gene Set Deregulation (Represented as Log_2 Fold Change) Following *o*-Dichlorobenzene Exposure of Each Gene Annotated to the Five MSigDB Supersets

Upregulated genes are displayed in green and downregulated genes in purple with color density indicating the relative strength of the change.

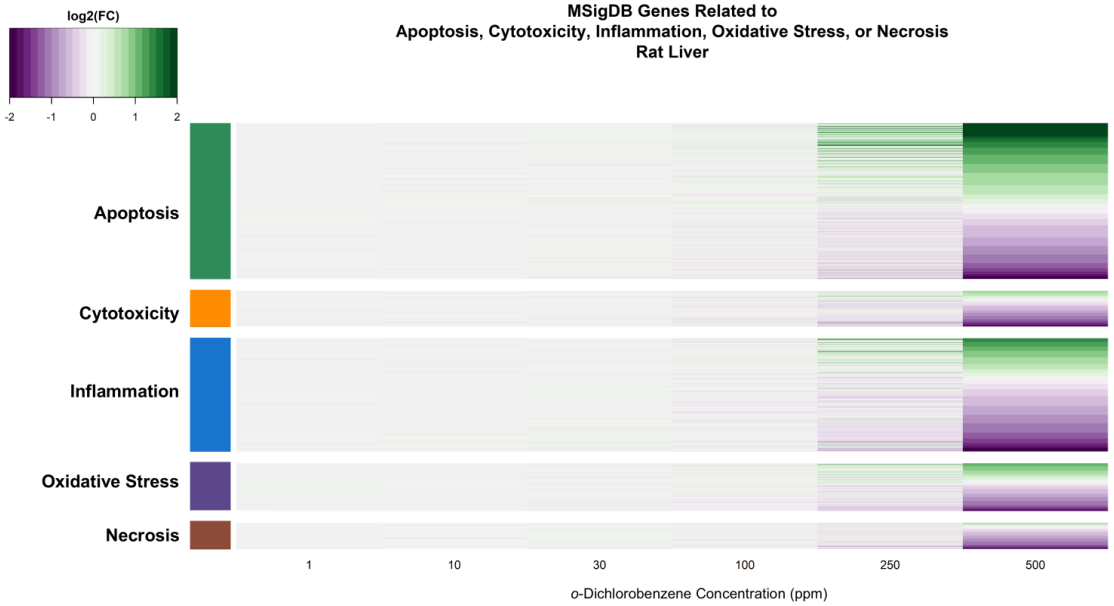

Figure 4-8. Heatmap of the Rat Liver Gene Set Deregulation (Represented as Log2 Fold Change) Following o-Dichlorobenzene Exposure of Each Gene Annotated to the Five MSigDB Supersets

Upregulated genes are displayed in green and downregulated genes in purple with color density indicating the relative strength of the change**.**

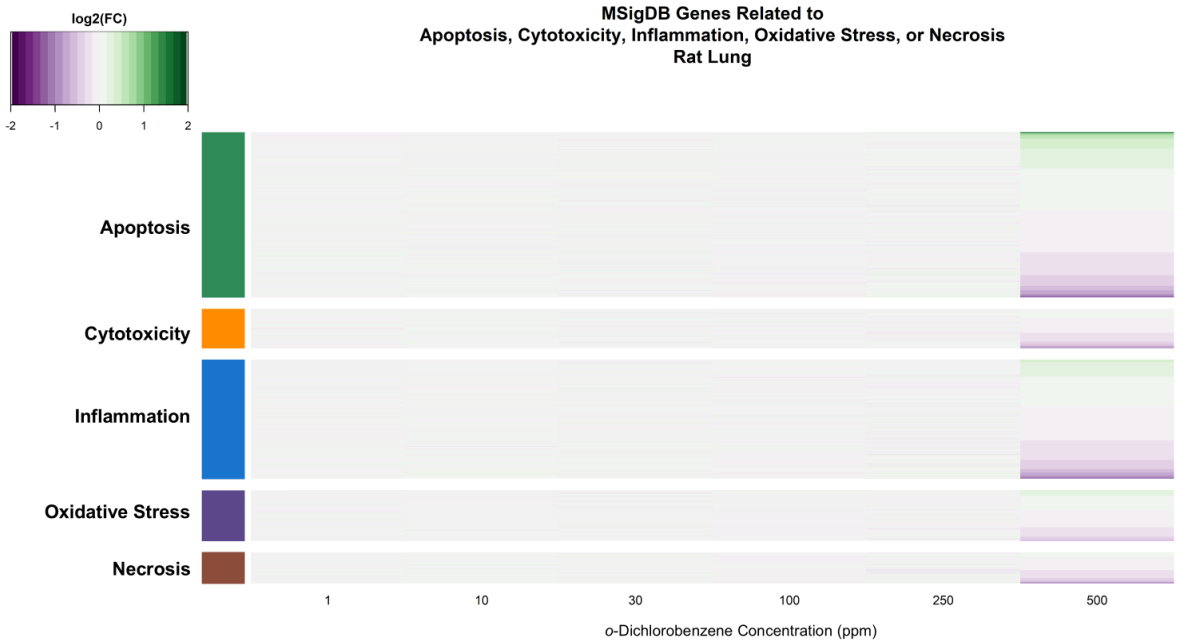

Figure 4-9. Heatmap of the Rat Lung Gene Set Deregulation (Represented as Log_2 Fold Change) Following *o*-Dichlorobenzene Exposure of Each Gene Annotated to the Five MSigDB Supersets

Upregulated genes are displayed in green and downregulated genes in purple with color density indicating the relative strength of the change.

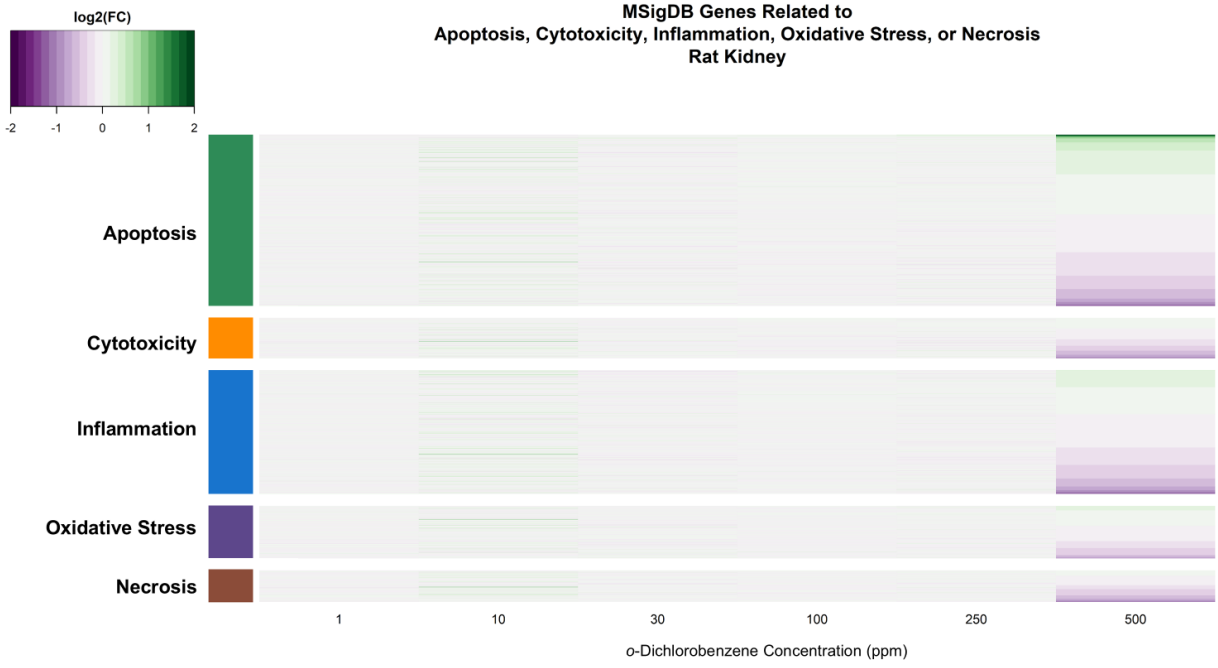

Figure 4-10. Heatmap of the Rat Kidney Gene Set Deregulation (Represented as Log_2 Fold Change) Following *o*-Dichlorobenzene Exposure of Each Gene Annotated to the Five MSigDB Supersets

Upregulated genes are displayed in green and downregulated genes in purple with color density indicating the relative strength of the change.

4.2.3 tPOD Determination Analysis for *o*-Dichlorobenzene

BMD analysis was performed on the individual genes and collective gene sets that were identified through the different gene expression analysis approaches described in Appendix A.5, with the GOBP gene set, the full MSigDB gene set, and each MSigDB superset relating to apical effects individually and using the aggregated superset defined by gene sets containing any of the five superset terms (apoptosis, cytotoxicity, inflammation, oxidative stress, necrosis) of interest. A summary of the different tPOD derivation methods used by EPA in this TSD can be found in Appendix A.5. In brief, three curated databases of gene sets were utilized: (1) GOBP, (2) MSigDB, and (3) a mechanism-informed curated subset of MSigDB termed "supersets." Two analytical approaches were used, one that selected a lowest (*i.e.,* most sensitive) value in the distribution of gene sets within each database, representing a highly conservative POD estimate; and another that selected the 5th percentile of the distribution of values, which aligns with established EPA risk assessment approaches such as for deriving numerical national water quality criteria (U.S. EPA, 1985), the Threshold of Toxicological Concern (Kroes et al., 2005), and species sensitivity distribution analysis (Dhond and Barron, 2022; Newman et al., 2000).

Furthermore, within i*n vitro* NAMs, the lower 5th percentile of the distribution of benchmark dose values has been used to identify PODs based on preliminary chemical bioactivity screening (Friedman et al., 2020), and, more broadly, in the field of toxicogenomic research using the distribution of benchmark doses of gene sets in multiple *in vitro* high-throughput transcriptomic analyses (Bundy et al., 2024; Harrill et al., 2024; Harrill et al., 2021). Because the 5th percentile gene set BMDL median was below, but within approximately an order of magnitude of, the inflection point of the BMDL median accumulation plot curves, EPA determined that this dose most closely approximated the BMDL median just prior to maximal pathway activation and thus was able to capture more sensitive (yet not overly sensitive) gene set expression changes. In addition, the MSigDB gene sets used to derive tPODs are often defined from experimental or computational studies establishing functional effects at the cellular level or in some cases, as with *in vivo* genetic knockouts, organism level. Custom curated lists of these gene sets can be identified that provide a means to anchor changes in these gene sets with specific phenotypes/mechanisms of interest based on the available hazard database.

1. The $GOBP_{low}$ tPOD was summarized as the BMDL of the lowest median BMD GOBP gene set with at least three genes and follows the *Standard Methods for Development of EPA Transcriptomic Assessment Products* (U.S. EPA, 2024b).
2. The $MSigDB_{low}$ tPOD was calculated considering the full landscape of MSigDB gene sets with at least three genes and summarized as the lowest median BMDL MSigDB gene set.
3. The MSigDB $SUPER_{low}$ tPOD was calculated considering only superset-specific MSigDB gene sets with at least three genes and summarized as the lowest median BMDL MSigDB superset.
4. The $GOBP_{0.05}$ tPOD was summarized as the 5th percentile of the median BMDL gene set distribution from the GOBP gene set with at least three genes.
5. The $MSigDB_{0.05}$ tPOD was summarized as the 5th percentile of the median BMDL gene set distribution from the full landscape of MSigDB gene sets with at least three genes.
6. The MSigDB $SUPER_{0.05}$ tPOD was summarized as the 5th percentile of the median BMDL superset distribution from the MSigDB supersets with at least three genes.

Within the mouse liver, the $GOBP_{low}$ tPOD was 1.22 ppm for the GOBP term "cellular response to cholesterol," which represents the tPOD estimate following the *Standard Methods for Development of EPA Transcriptomic Assessment Products* (U.S. EPA, 2024b). EPA emphasizes that the description of the gene set underlying this tPOD is not necessarily relevant to the underlying biology, mechanism of

toxicity, or observed apical phenotypes from *o*-dichlorobenzene exposure. Rather, it represents a highly conservative POD that is not linked to any specific apical effect, unlike the MSigDB superset-based methods EPA presents in this draft TSD. A comprehensive background, methods, analysis, and description of the $GOBP_{low}$ tPODs across tissues and species following the *Standard Methods for Development of EPA Transcriptomic Assessment Products* (U.S. EPA, 2024b) can be found in Appendix B and will not be described here. Within the combined set of aggregated supersets, the MSigDB $SUPER_{low}$ was 1.49 ppm for the GOBP term "Pyroptotic inflammatory response." The MSigDB $SUPER_{0.05}$ BMDL Median was 29.87 ppm. A summary of the mouse liver BMDL Median results for the five MSigDB gene sets can be found in Table 4-1 and accumulation plots for each of the selected MSigDB gene sets compared with the full landscape of gene sets in MSigDB are shown in Figure 4-11 (MSigDB $SUPER_{low}$) and Figure 4-12 (MSigDB $SUPER_{0.05}$ or the specific 5th percentile tPOD for the five individual supersets).

Table 4-1. Benchmark Doses Associated with the Enrichment of Gene Expression Relating to Apical Effects from *o*-Dichlorobenzene Exposure (Apoptosis, Cytotoxicity, Inflammation, Oxidative Stress, Necrosis) Within Mouse Liver

	Pathway Accession	Lowest BMDL Median[a] (ppm)	5th Percentile BMDL Median[b] (ppm)
GOBP	GO:0071397	1.22	35.12
MSigDB[c]	MM3840	0.96	37.58
Combined MSigDB Superset[d]	MM1505	1.49 (cytotoxicity)	29.87
MSigDB cytotoxicity	MM1505	1.49	11.80
MSigDB apoptosis	MM11665, MM14251	5.74	35.68
MSigDB inflammation	MM14251	5.74	12.54
MSigDB oxidative stress	MM10820	37.58	44.16
MSigDB necrosis	MM13035, MM13818, MM13053	38.93	38.93

[a] The lowest BMDL median for GOBP represents the BMDL of the lowest GO term rank ordered by BMD Median.
[b] The 5th percentile BMDL Median for GOBP represents the 5th percentile of GO terms rank ordered by BMDL Median.
[c] The full MSigDB collection of gene sets.
[d] The Combined MSigDB Superset represents a consensus of multiple individual gene sets associated with a specific biological response. For this evaluation, cytotoxicity, apoptosis, inflammation, oxidative stress, and necrosis were prioritized.
The MSigDB and MSigDB superset results presented here are rank ordered by gene set-level BMDL Median and report the lowest and distribution-based 5th percentile gene set BMDL Medians for comparison.
For gene set accessions, the "GO" prefix refers to "Gene Ontology" accession identifiers, while the "MM" prefix refers to "mouse MSigDB" accession identifiers.

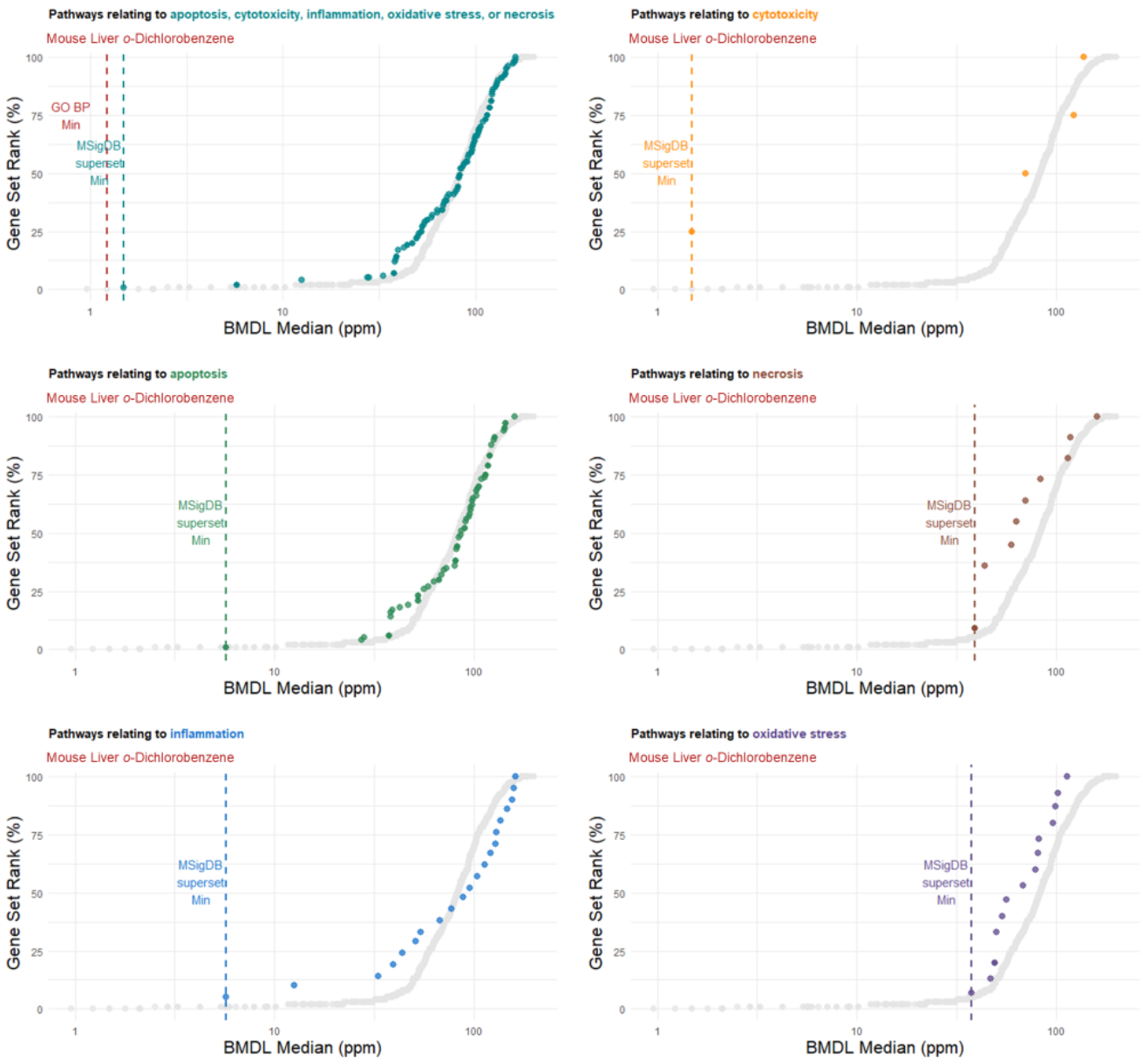

Figure 4-11. Mouse Liver Accumulation Plots Showing the Gene Set Ranks (%)
All gene sets in the MSigDB database are shown with grayed circles; each circle represents a distinct MSigDB gene set term. The combined gene set with any term from apoptosis, cytotoxicity, inflammation, necrosis, and oxidative stress, and including at least three genes with BMD meeting all inclusion criteria, is shown against the landscape of all gene sets in color, with each subsequent plot highlighting a specific subset of those gene sets. The vertical dashed red line highlights the $GOBP_{low}$ tPOD, the others highlight either the MSigDB $SUPER_{low}$ tPOD or the specific lower BMDL Median tPOD for the five individual supersets.

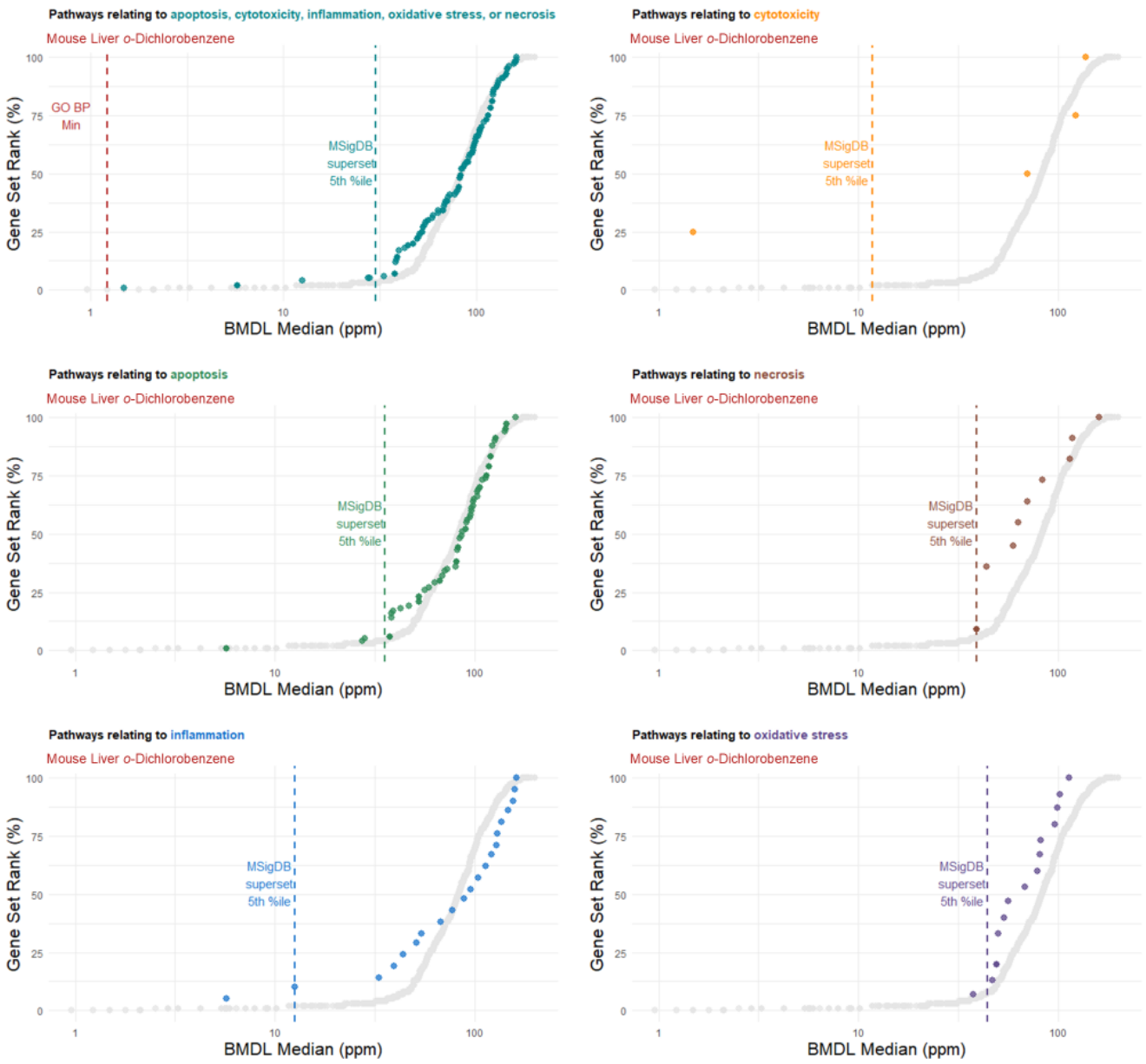

Figure 4-12. Mouse Liver Accumulation Plots Showing the Gene Set Ranks (%)
All gene sets in the MSigDB database are shown with grayed circles; each circle represents a distinct MSigDB gene set term. The combined gene set with any term from apoptosis, cytotoxicity, inflammation, necrosis, and oxidative stress, and including at least three genes with BMD meeting all inclusion criteria, is shown against the landscape of all gene sets in color, with each subsequent plot highlighting a specific subset of those gene sets. The vertical dashed red line highlights the $GOBP_{low}$ tPOD, the others highlight either the MSigDB $SUPER_{0.05}$ tPOD or the specific 5th percentile tPOD for the five individual supersets.

Within the aggregated supersets, the MSigDB $SUPER_{low}$ was 1.98 ppm for the GOBP pathway "T-cell differentiation involved in immune response." The MSigDB $SUPER_{0.05}$ tPOD BMDL Median was 6.67 ppm. A full table of mouse lung BMD results for the five MSigDB supersets can be found in Table 4-2, and accumulation plots for each of the selected MSigDB gene sets compared with the full landscape of gene sets in MSigDB are shown in Figure 4-13 (MSigDB $SUPER_{low}$) and Figure 4-14 (MSigDB $SUPER_{0.05}$ or the specific 5th percentile tPOD for the 5 individual supersets).

Table 4-2. BMD Modeling Results of Targeted Gene Sets Relating to Apical Effects from *o*-Dichlorobenzene Exposure (Apoptosis, Cytotoxicity, Inflammation, Oxidative Stress, Necrosis) Within Mouse Lung

	Gene Set Accession	Lowest BMDL Median[a] (ppm)	5th Percentile BMDL Median[b] (ppm)
GOBP	GO:0001843	1.21	9.59
MSigDB[c]	GO:0009142	1.07	7.23
MSigDB Combined Superset[d]	MM9945, MM4226	1.98 (cytotoxicity)	6.67
MSigDB cytotoxicity	MM4226	1.98	7.21
MSigDB inflammation	MM9945	1.98	2.05
MSigDB necrosis	MM5039	6.67	17.05
MSigDB apoptosis	MM977	10.51	23.83
MSigDB oxidative stress	MM15941	11.06	29.84

[a] The lowest BMDL median for GOBP represents the BMDL of the lowest GO term rank ordered by BMD Median.
[b] The 5th percentile BMDL Median for GOBP represents the 5th percentile of GO terms rank ordered by BMDL Median.
[c] The full MSigDB collection of gene sets.
[d] The Combined MSigDB Superset represents a consensus of multiple individual gene sets associated with a specific biological response. For this evaluation, cytotoxicity, apoptosis, inflammation, oxidative stress, and necrosis were prioritized.
The MSigDB and MSigDB superset results presented here are rank ordered by gene set-level BMDL Median and report the lowest and distribution-based 5th percentile gene set BMDL Medians for comparison.
For gene set accessions, the "GO" prefix refers to "Gene Ontology" accession identifiers, while the "MM" prefix refers to "mouse MSigDB" accession identifiers.

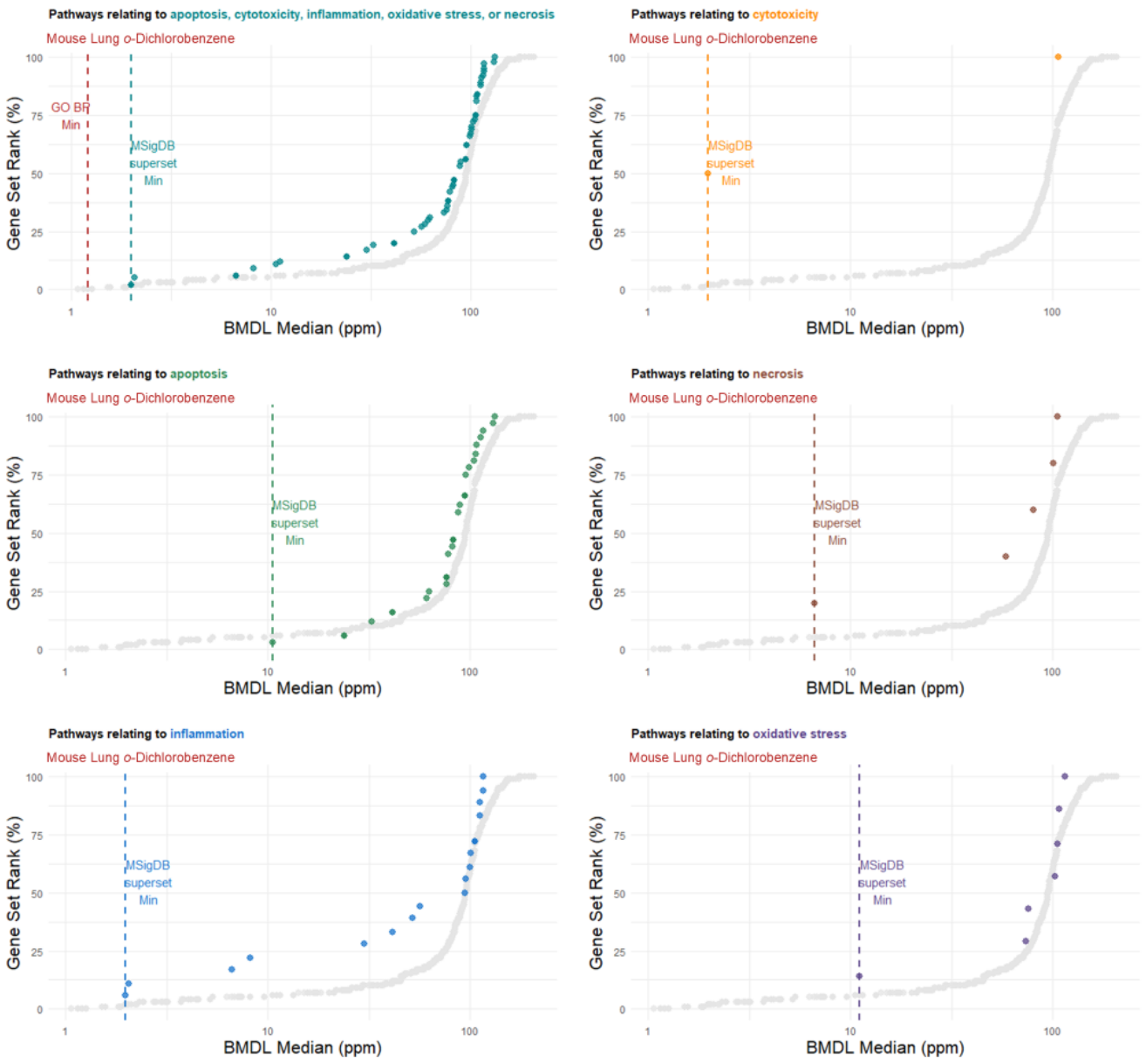

Figure 4-13. Mouse Lung Accumulation Plots Showing the Gene Set Ranks (%)
All gene sets in the MSigDB database are shown with grayed circles; each circle represents a distinct MSigDB gene set term. The combined gene set with any term from apoptosis, cytotoxicity, inflammation, necrosis, and oxidative stress, and including at least three genes with BMD meeting all inclusion criteria, is shown against the landscape of all gene sets in color, with each subsequent plot highlighting a specific subset of those gene sets. The vertical dashed red line highlights the $GOBP_{low}$ tPOD, the others highlight either the MSigDB $SUPER_{low}$ tPOD or the specific lower BMDL Median tPOD for the five individual supersets.

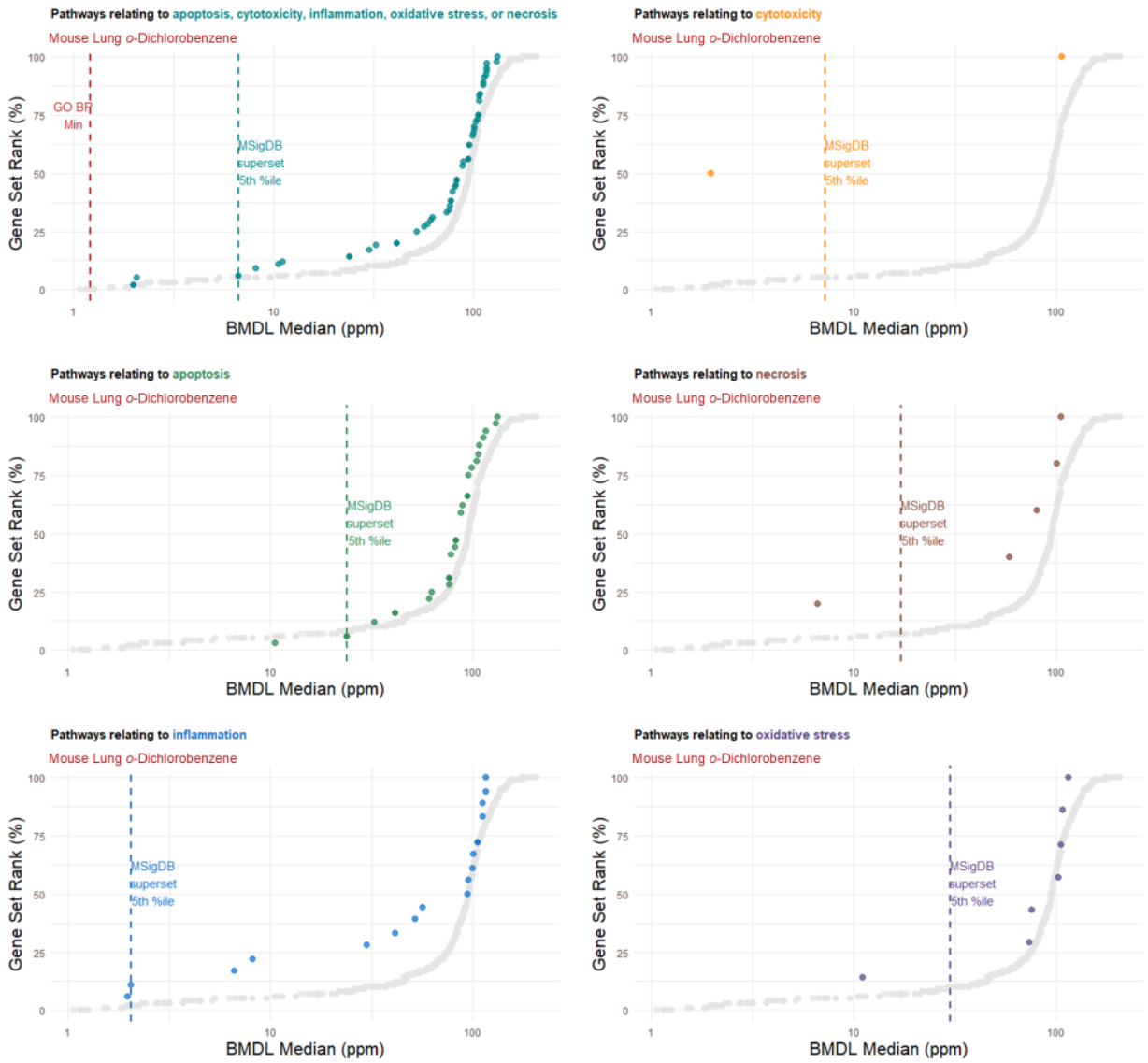

Figure 4-14. Mouse Lung Accumulation Plots Showing the Gene Set Ranks (%)
All gene sets in the MSigDB database are shown with grayed circles; each circle represents a distinct MSigDB gene set term. The combined gene set with any term from apoptosis, cytotoxicity, inflammation, necrosis, and oxidative stress, and including at least three genes with BMD meeting all inclusion criteria, is shown against the landscape of all gene sets in color, with each subsequent plot highlighting a specific subset of those gene sets. The vertical dashed red line highlights the $GOBP_{low}$ tPOD, the others highlight either the MSigDB $SUPER_{0.05}$ tPOD or the specific 5th percentile tPOD for the five individual supersets.

Within the combined set of aggregated supersets, the MSigDB $SUPER_{low}$ was 2.12 ppm for the WikiPathways term "Oxidative stress and REDOX." The MSigDB $SUPER_{0.05}$ BMDL Median tPOD was 4.72 ppm. A full table of mouse kidney BMD results for the five MSigDB gene sets can be found in Table 4-3, and accumulation plots for each of the selected MSigDB gene sets compared with the full landscape of gene sets in MSigDB are shown in Figure 4-15 (MSigDB $SUPER_{low}$) and Figure 4-16 (MSigDB $SUPER_{0.05}$ or the specific 5th percentile tPOD for the 4 individual supersets). Note that there were no cytotoxicity-related gene sets identified in mouse kidney.

Table 4-3. BMD Modeling Results of Targeted Gene Sets Relating to Apical Effects from *o*-Dichlorobenzene Exposure (Apoptosis, Cytotoxicity, Inflammation, Oxidative Stress, Necrosis) Within Mouse Kidney

	Gene Set Accession	Lowest BMDL Median[a] (ppm)	5th Percentile BMDL Median[b] (ppm)
GOBP	GO:0007623	1.01	6.20
MSigDB[c]	M2581	0.74	6.18
MSigDB Combined Superset[d]	MM15823	2.12 (oxidative stress)	4.72
MSigDB oxidative stress	MM15823	2.12	8.68
MSigDB inflammation	MM9519	3.44	21.71
MSigDB apoptosis	MM11663	4.72	15.56
MSigDB necrosis	MM6985	4.72	9.68

[a] The lowest BMDL median for GOBP represents the BMDL of the lowest GO term rank ordered by BMD Median.
[b] The 5th percentile BMDL Median for GOBP represents the 5th percentile of GO terms rank ordered by BMDL Median.
[c] The full MSigDB collection of gene sets.
[d] The Combined MSigDB Superset represents a consensus of multiple individual gene sets associated with a specific biological response. For this evaluation, cytotoxicity, apoptosis, inflammation, oxidative stress, and necrosis were prioritized.
The MSigDB and MSigDB superset results presented here are rank ordered by gene set-level BMDL Median and report the lowest and distribution-based 5th percentile gene set BMDL Medians for comparison.
For gene set accessions, the "GO" prefix refers to "Gene Ontology" accession identifiers, while the "MM" prefix refers to "mouse MSigDB" accession identifiers.

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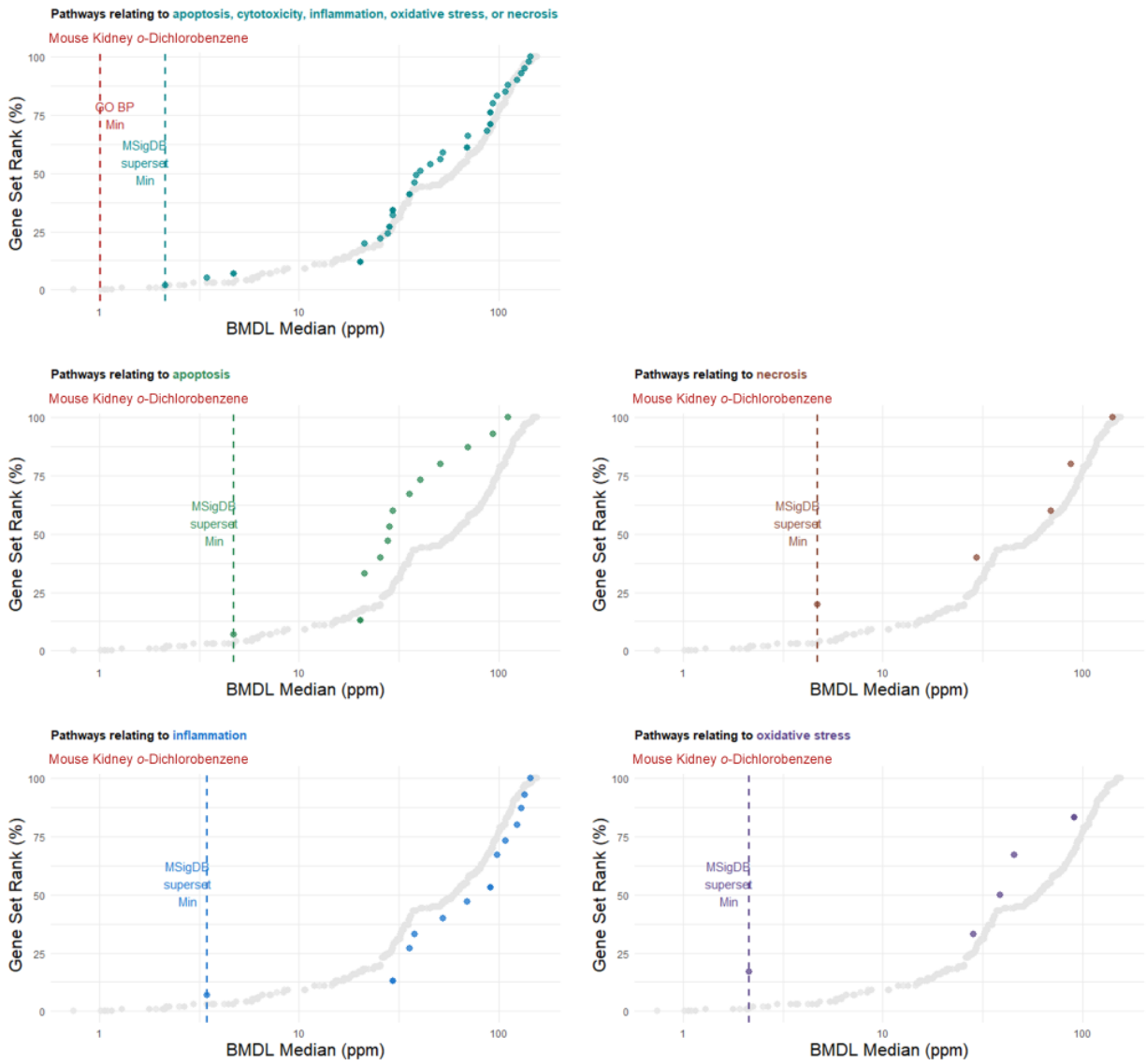

Figure 4-15. Mouse Kidney Accumulation Plots Showing the Gene Set Ranks (%)
All gene sets in the MSigDB database are shown with grayed circles; each circle represents a distinct MSigDB gene set term. The combined gene set with any term from apoptosis, cytotoxicity, inflammation, necrosis, and oxidative stress, and including at least three genes with BMD meeting all inclusion criteria, is shown against the landscape of all gene sets in color, with each subsequent plot highlighting a specific subset of those gene sets. The vertical dashed red line highlights the $GOBP_{low}$ tPOD, the others highlight either the MSigDB $SUPER_{low}$ tPOD or the specific lower BMDL Median tPOD for the four individual supersets. Note that there were no cytotoxicity-related gene sets identified in mouse kidney.

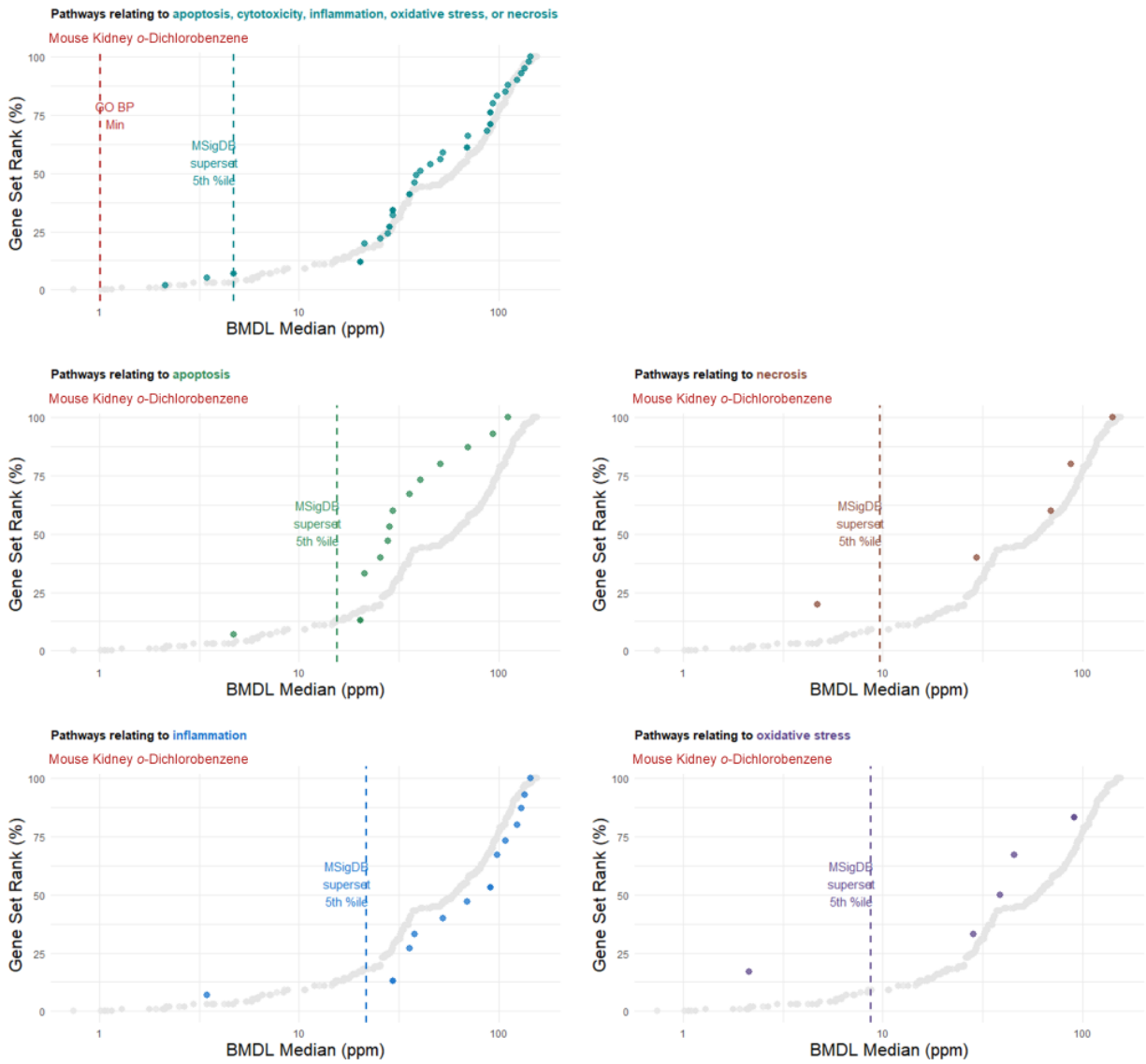

Figure 4-16. Mouse Kidney Accumulation Plots Showing the Gene Set Ranks (%)
All gene sets in the MSigDB database are shown with grayed circles; each circle represents a distinct MSigDB gene set term. The combined gene set with any term from apoptosis, cytotoxicity, inflammation, necrosis, and oxidative stress, and including at least three genes with BMD meeting all inclusion criteria, is shown against the landscape of all gene sets in color, with each subsequent plot highlighting a specific subset of those gene sets. The vertical dashed red line highlights the $GOBP_{low}$ tPOD, the others highlight either the MSigDB $SUPER_{0.05}$ tPOD or the specific 5th percentile tPOD for the four individual supersets. Note that there were no cytotoxicity-related gene sets identified in mouse kidney.

Within the combined set of curated gene sets, the MSigDB $SUPER_{low}$ was 40.38 ppm for the GOBP pathway "bleb assembly." The MSigDB $SUPER_{0.05}$ BMDL Median was 112.49 ppm. A full table of rat liver BMD results for the five gene sets can be found in Table 4-4, and accumulation plots for each of the selected gene sets compared with the full landscape of gene sets in MSigDB are shown in Figure 4-17 (MSigDB $SUPER_{low}$) and Figure 4-18 (MSigDB $SUPER_{0.05}$ or the specific 5th percentile tPOD for the 5 individual supersets).

Table 4-4. BMD Modeling Results of Targeted Gene Sets Relating to Apical Effects from *o*-Dichlorobenzene Exposure (Apoptosis, Cytotoxicity, Inflammation, Oxidative Stress, Necrosis) Within Rat Liver

	Gene Set Accession	Lowest BMDL Median (ppm) [a]	5th Percentile BMDL Median[b] (ppm)
GOBP	GO:0032467	41.10	107.95
MSigDB[c]	GO:1904668	39.29	106.48
MSigDB Combined Superset[d]	M23053	40.38 (apoptosis)	112.49
MSigDB apoptosis	M23053	40.38	113.21
MSigDB cytotoxicity	M22203	74.96	93.26
MSigDB inflammation	M14283	91.09	128.58
MSigDB oxidative stress	M19540	104.27	108.43
MSigDB necrosis	M29666	168.02	171.77

[a] The lowest BMDL median for GOBP represents the BMDL of the lowest GO term rank ordered by BMD Median.
[b] The 5th percentile BMDL Median for GOBP represents the 5th percentile of GO terms rank ordered by BMDL Median.
[c] The full MSigDB collection of gene sets.
[d] The Combined MSigDB Superset represents a consensus of multiple individual gene sets associated with a specific biological response. For this evaluation, cytotoxicity, apoptosis, inflammation, oxidative stress, and necrosis were prioritized.
The MSigDB and MSigDB superset results presented here are rank ordered by gene set-level BMDL Median and report the lowest and distribution-based 5th percentile gene set BMDL Medians for comparison.
For gene set accessions, the "GO" prefix refers to Gene Ontology accession identifiers, while the "M" prefix refers to MSigDB accession identifiers that were mapped via orthology from human gene sets since there are no native rat gene sets available in MSigDB.

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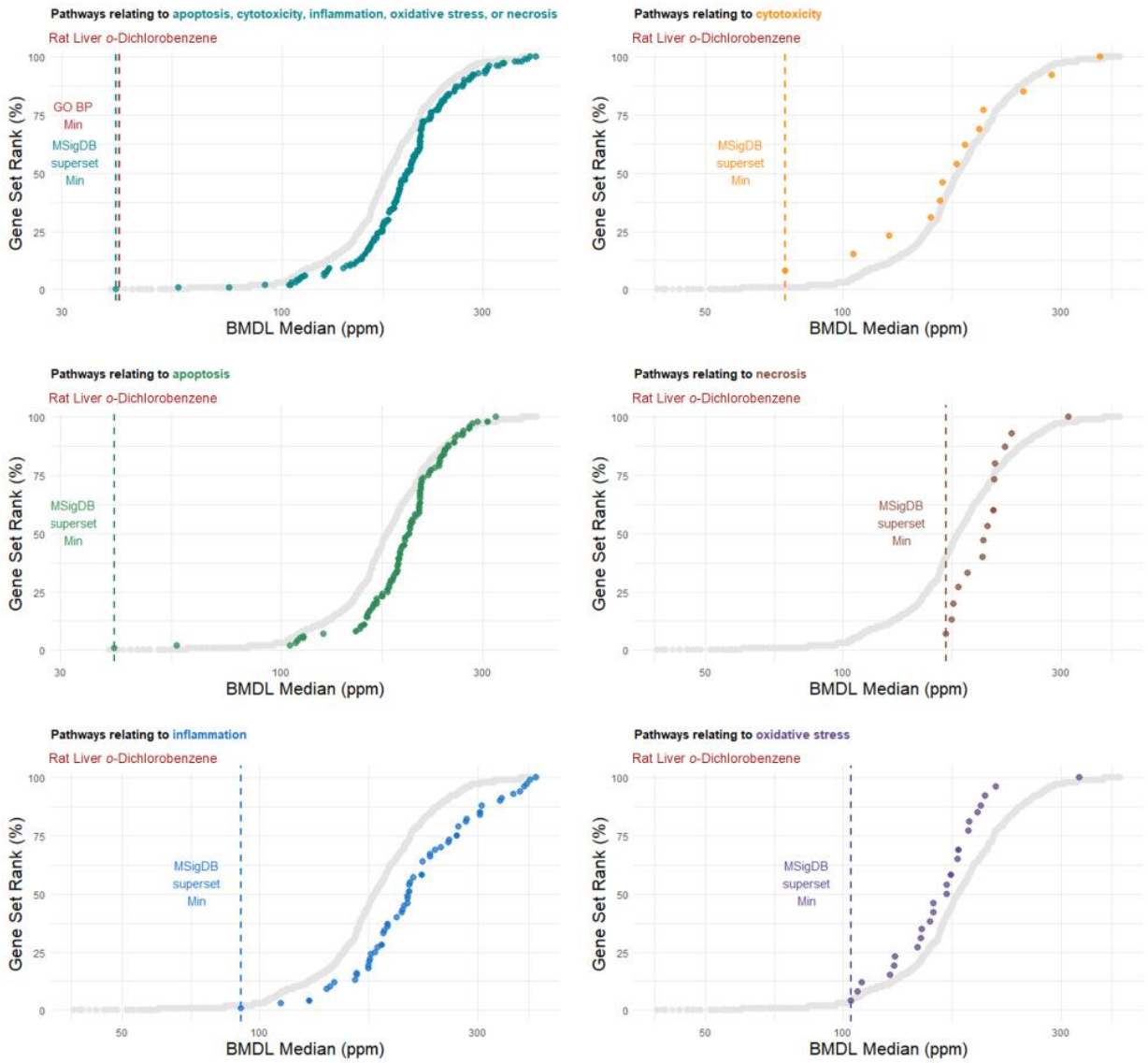

Figure 4-17. Rat Liver Accumulation Plots Showing the Gene Set Ranks (%)
All gene sets in the MSigDB database are shown with grayed circles; each circle represents a distinct MSigDB gene set term. The combined gene set with any term from apoptosis, cytotoxicity, inflammation, necrosis, and oxidative stress, and including at least three genes with BMD meeting all inclusion criteria, is shown against the landscape of all gene sets in color, with each subsequent plot highlighting a specific subset of those gene sets. The vertical dashed red line highlights the $GOBP_{low}$ tPOD, the others highlight either the MSigDB $SUPER_{low}$ tPOD or the specific lower BMDL Median tPOD for the five individual supersets.

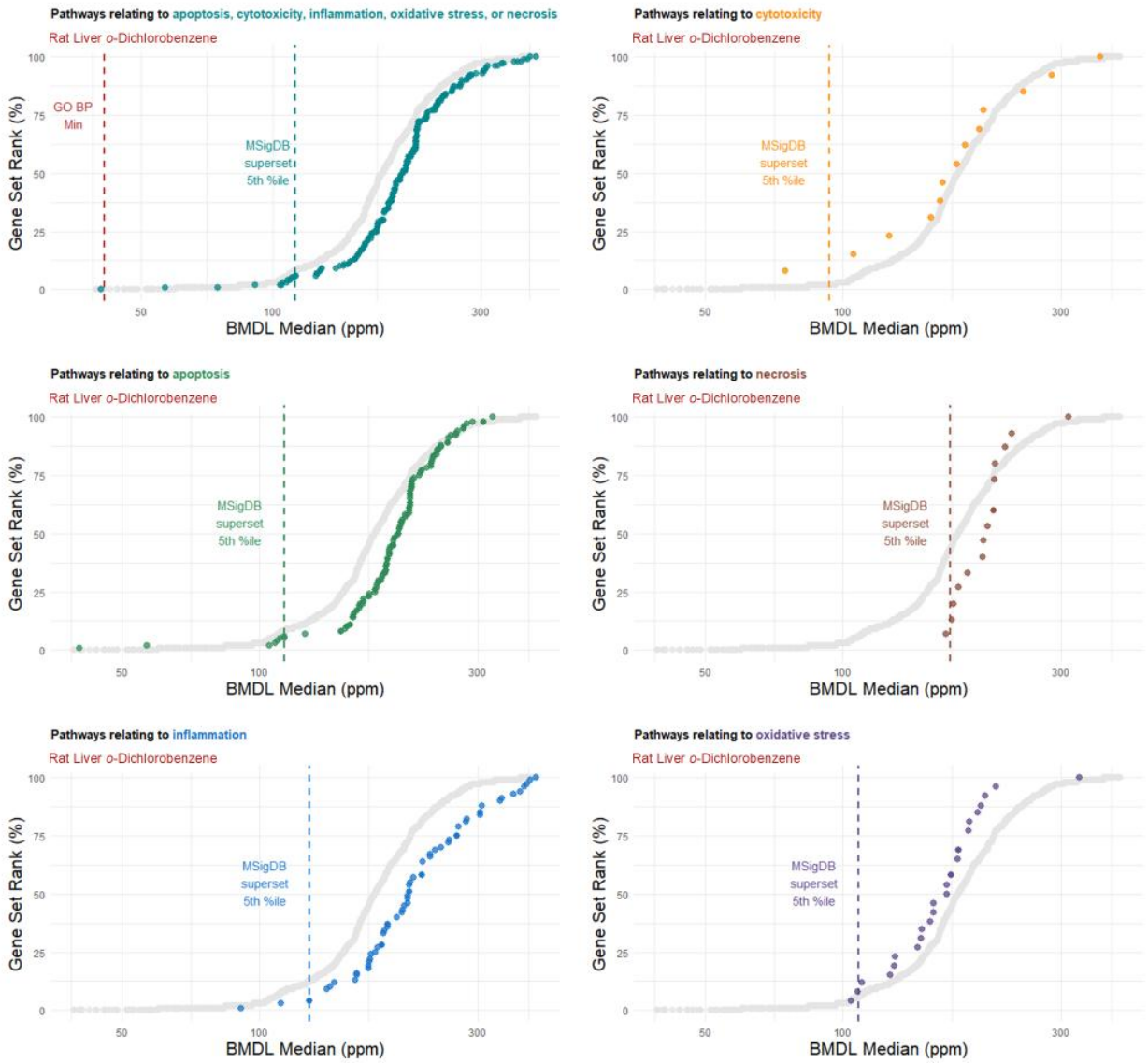

Figure 4-18. Rat Liver Accumulation Plots Showing the Gene Set Ranks (%)
All gene sets in the MSigDB database are shown with grayed circles; each circle represents a distinct MSigDB gene set term. The combined gene set with any term from apoptosis, cytotoxicity, inflammation, necrosis, and oxidative stress, and including at least three genes with BMD meeting all inclusion criteria, is shown against the landscape of all gene sets in color, with each subsequent plot highlighting a specific subset of those gene sets. The vertical dashed red line highlights the $GOBP_{low}$ tPOD, the others highlight either the MSigDB $SUPER_{0.05}$ tPOD or the specific 5th percentile tPOD for the five individual supersets.

Within the combined set of curated gene sets, the MSigDB $SUPER_{low}$ was 200 ppm for the GOBP pathway "JNK cascade." The MSigDB $SUPER_{0.05}$ BMDL Median tPOD was 218.79 ppm. A full table of rat lung BMD results for the five gene sets can be found in Table 4-5, and accumulation plots for each of the selected gene sets compared with the full landscape of gene sets in MSigDB are shown in Figure 4-19 (MSigDB $SUPER_{low}$) and Figure 4-20 (MSigDB $SUPER_{0.05}$ or the specific 5th percentile tPOD for the 5 individual supersets).

Table 4-5. BMD Modeling Results of Targeted Gene Sets Relating to Apical Effects from *o*-Dichlorobenzene Exposure (Apoptosis, Cytotoxicity, Inflammation, Oxidative Stress, Necrosis) Within Rat Lung

	Gene Set Accession	Lowest BMDL Median[a] (ppm)	5th Percentile BMDL Median[b] (ppm)
GOBP	GO:0016052	205.00	262.08
MSigDB[c]	M8994	175.2	262.08
MSigDB Combined Superset[d]	M13069	200.00 (apoptosis, inflammation)	218.79
MSigDB apoptosis	M13069	200.00	251.56
MSigDB inflammation	M13069	200.00	204.55
MSigDB necrosis	M16514	216.56	223.30
MSigDB oxidative stress	M15990	245.09	256.36
MSigDB cytotoxicity	M8134	371.26	371.60

[a] The lowest BMDL median for GOBP represents the BMDL of the lowest GO term rank ordered by BMD Median.
[b] The 5th percentile BMDL Median for GOBP represents the 5th percentile of GO terms rank ordered by BMDL Median.
[c] The full MSigDB collection of gene sets.
[d] The Combined MSigDB Superset represents a consensus of multiple individual gene sets associated with a specific biological response. For this evaluation, cytotoxicity, apoptosis, inflammation, oxidative stress, and necrosis were prioritized.
The MSigDB and MSigDB superset results presented here are rank ordered by gene set-level BMDL Median and report the lowest and distribution-based 5th percentile gene set BMDL Medians for comparison.
For gene set accessions, the "GO" prefix refers to Gene Ontology accession identifiers, while the "M" prefix refers to MSigDB accession identifiers that were mapped via orthology from human gene sets since there are no native rat gene sets available in MSigDB.

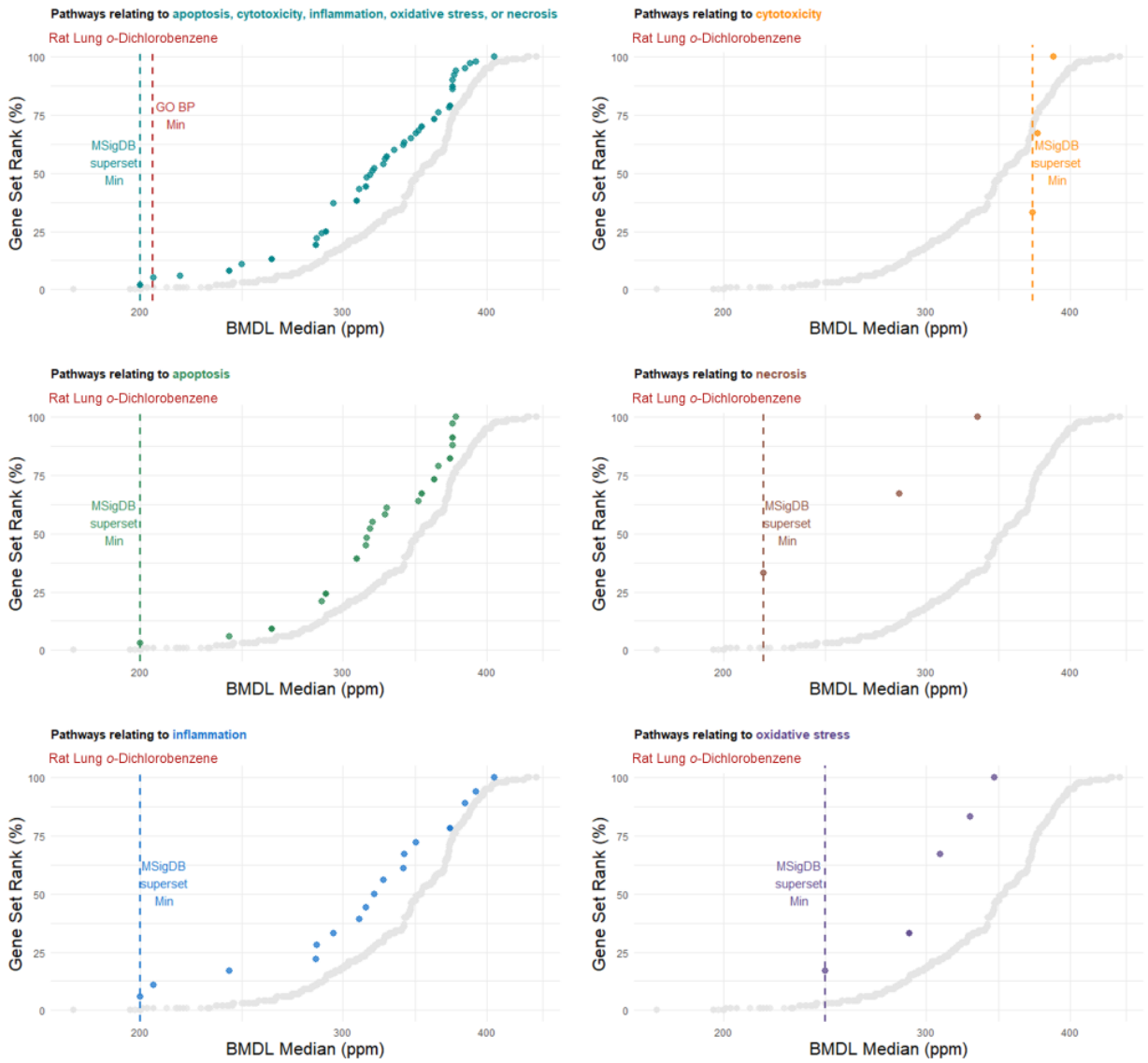

Figure 4-19. Rat Lung Accumulation Plots Showing the Gene Set Ranks (%)
All gene sets in the MSigDB database are shown with grayed circles; each circle represents a distinct MSigDB gene set term. The combined gene set with any term from apoptosis, cytotoxicity, inflammation, necrosis, and oxidative stress, and including at least three genes with BMD meeting all inclusion criteria, is shown against the landscape of all gene sets in color, with each subsequent plot highlighting a specific subset of those gene sets. The vertical dashed red line highlights the $GOBP_{low}$ tPOD, the others highlight either the MSigDB $SUPER_{low}$ tPOD or the specific lower BMDL Median tPOD for the five individual supersets.

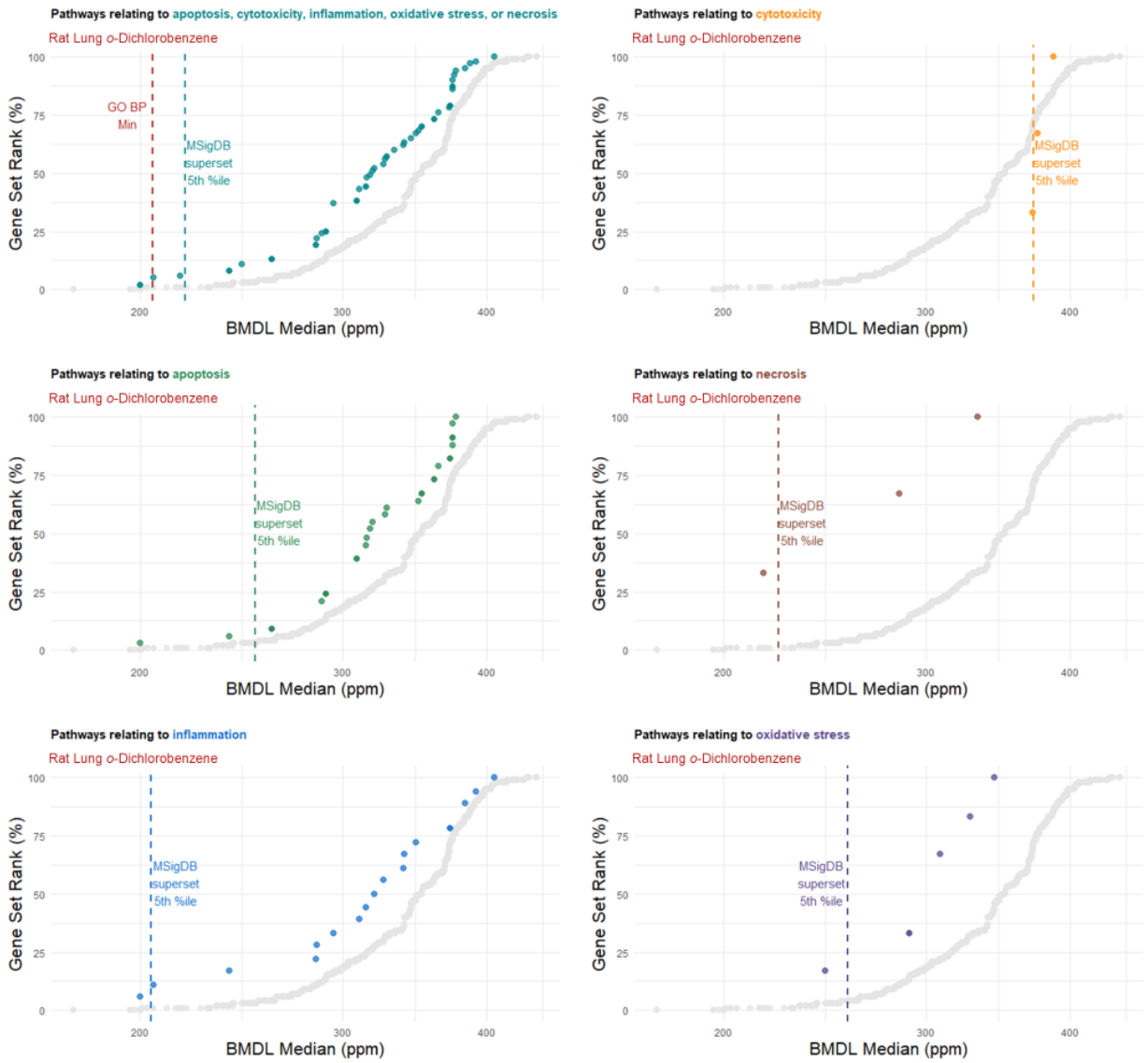

Figure 4-20. Rat Lung Accumulation Plots Showing the Gene Set Ranks (%)
All gene sets in the MSigDB database are shown with grayed circles; each circle represents a distinct MSigDB gene set term. The combined gene set with any term from apoptosis, cytotoxicity, inflammation, necrosis, and oxidative stress, and including at least three genes with BMD meeting all inclusion criteria, is shown against the landscape of all gene sets in color, with each subsequent plot highlighting a specific subset of those gene sets. The vertical dashed red line highlights the $GOBP_{low}$ tPOD, the others highlight either the MSigDB $SUPER_{0.05}$ tPOD or the specific 5th percentile tPOD for the five individual supersets.

Within the combined set of curated gene sets, the MSigDB $SUPER_{low}$ was 168.92 ppm for the Coates curated term "macrophage M1 vs M2 upregulated." The MSigDB $SUPER_{0.05}$ BMDL Median tPOD was 211.39 ppm. A full table of rat kidney BMD results for the five gene sets can be found in Table 4-6, and accumulation plots for each of the selected gene sets compared with the full landscape of gene sets in MSigDB are shown in Figure 4-21 (MSigDB $SUPER_{low}$) and Figure 4-22 (MSigDB $SUPER_{0.05}$ or the specific 5th percentile tPOD for the 5 individual supersets).

Table 4-6. BMD Modeling Results of Targeted Gene Sets Relating to Apical Effects from *o*-Dichlorobenzene Exposure (Apoptosis, Cytotoxicity, Inflammation, Oxidative Stress, Necrosis) Within Rat Kidney

	Gene Set Accession	Lowest BMDL Median[a] (ppm)	5th Percentile BMDL Median[b] (ppm)
GOBP	GO:0030522	116.00	235.36
MSigDB[c]	M29981	77.83	199.34
MSigDB Combined Superset[d]	M13671	168.92 (inflammation)	211.39
MSigDB inflammation	M13671	168.92	251.62
MSigDB apoptosis	M41716	178.62	218.49
MSigDB necrosis	M14642, M13837	182.80	182.80
MSigDB oxidative stress	M17410	207.03	223.93
MSigDB cytotoxicity	M8204	293.82	304.49

[a] The lowest BMDL median for GOBP represents the BMDL of the lowest GO term rank ordered by BMD Median.
[b] The 5th percentile BMDL Median for GOBP represents the 5th percentile of GO terms rank ordered by BMDL Median.
[c] The full MSigDB collection of gene sets.
[d] The Combined MSigDB Superset represents a consensus of multiple individual gene sets associated with a specific biological response. For this evaluation, cytotoxicity, apoptosis, inflammation, oxidative stress, and necrosis were prioritized.
The MSigDB and MSigDB superset results presented here are rank ordered by gene set-level BMDL Median and report the lowest and distribution-based 5th percentile gene set BMDL Medians for comparison.
For gene set accessions, the "GO" prefix refers to Gene Ontology accession identifiers, while the "M" prefix refers to MSigDB accession identifiers that were mapped via orthology from human gene sets since there are no native rat gene sets available in MSigDB.

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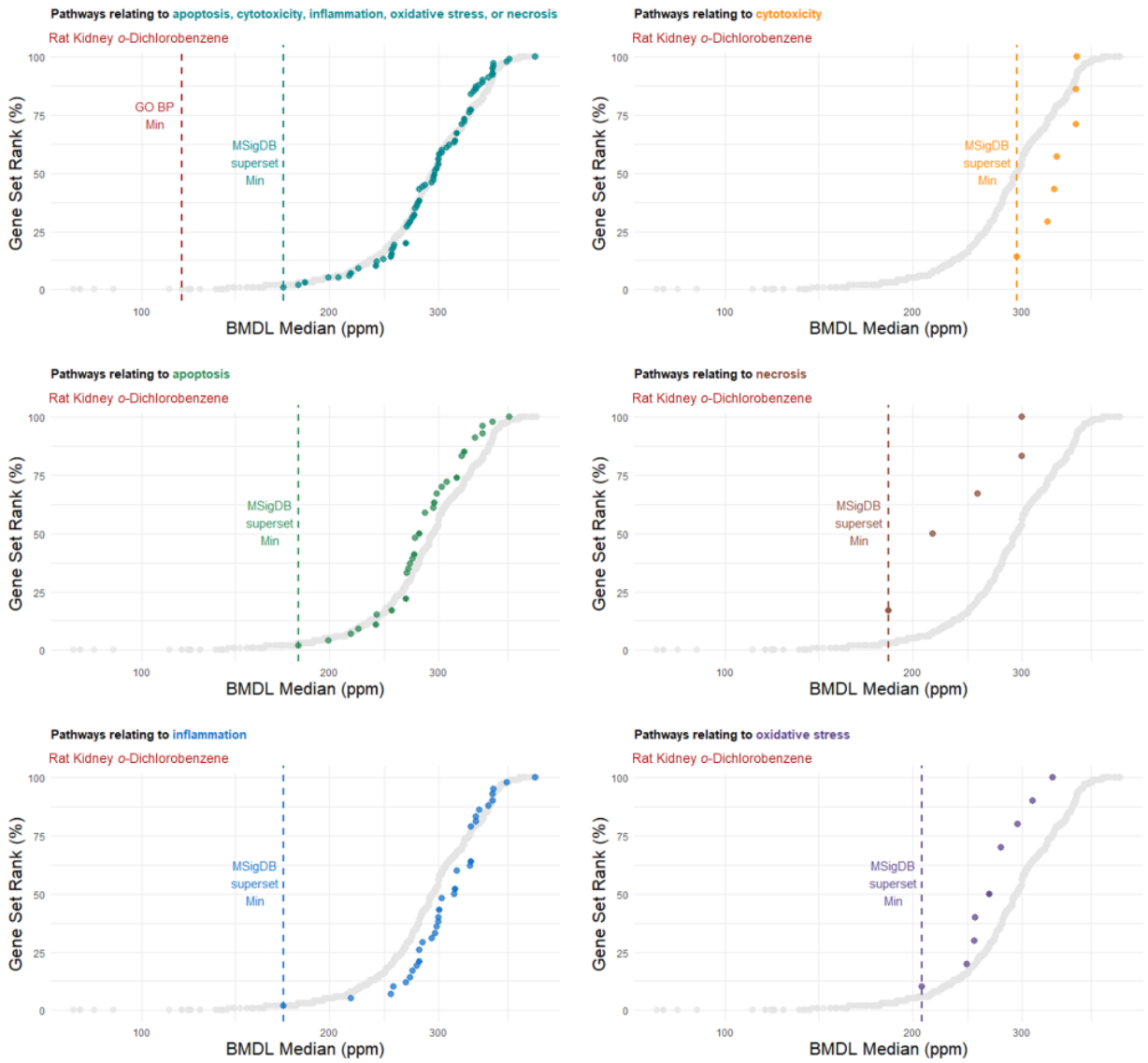

Figure 4-21. Rat Kidney Accumulation Plots Showing the Pathway Ranks (%)
All gene sets in the MSigDB database are shown with grayed circles; each circle represents a distinct MSigDB gene set term. The combined gene set with any term from apoptosis, cytotoxicity, inflammation, necrosis, and oxidative stress, and including at least three genes with BMD meeting all inclusion criteria, is shown against the landscape of all pathways in color, with each subsequent plot highlighting a specific subset of those pathways. The vertical dashed red line highlights the $GOBP_{low}$ tPOD, the others highlight either the MSigDB $SUPER_{low}$ tPOD or the specific lower BMDL Median tPOD for the five individual supersets.

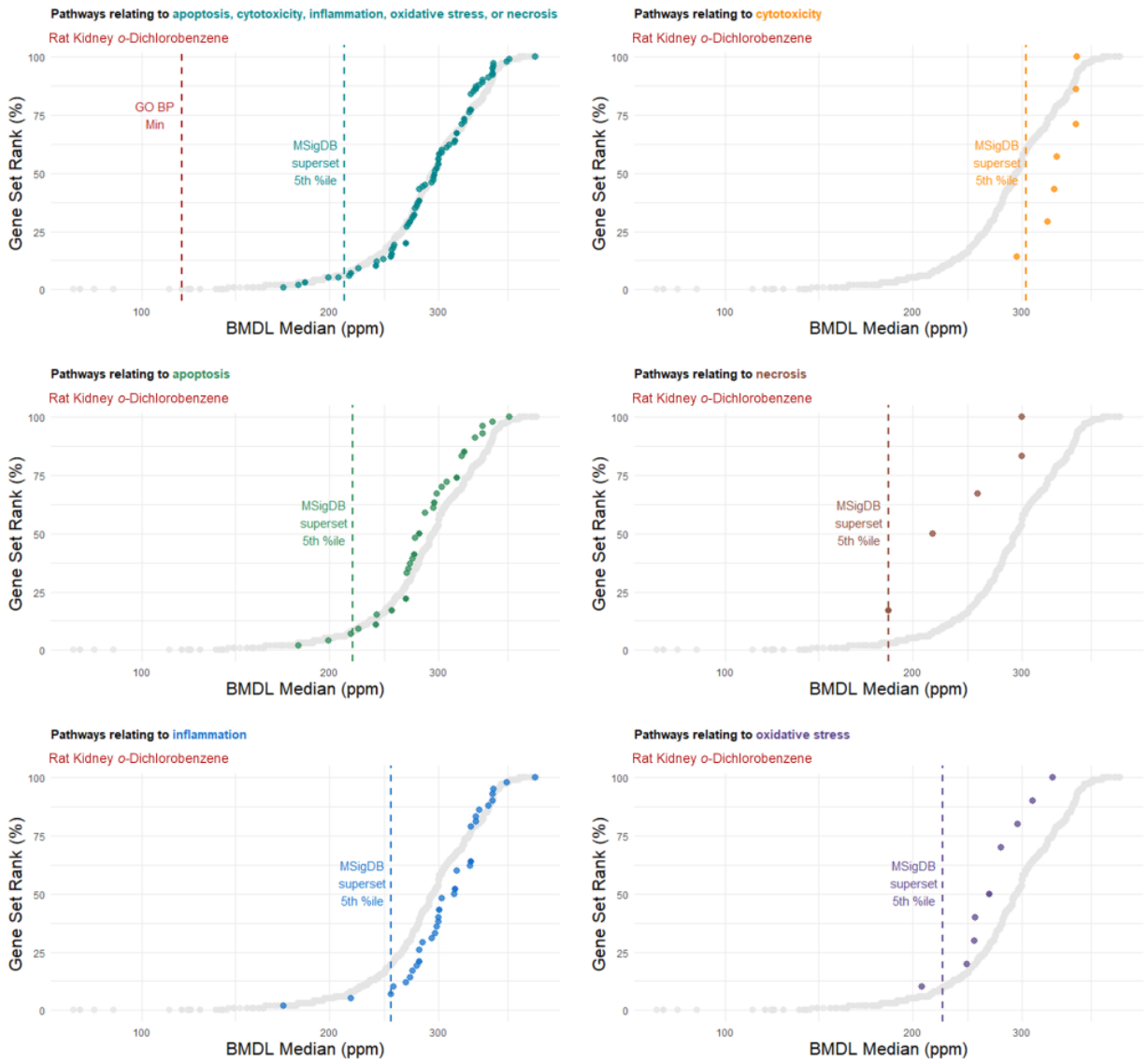

Figure 4-22. Rat Kidney Accumulation Plots Showing the Gene Set Ranks (%)
All gene sets in the MSigDB database are shown with grayed circles; each circle represents a distinct MSigDB gene set term. The combined gene set with any term from apoptosis, cytotoxicity, inflammation, necrosis, and oxidative stress, and including at least three genes with BMD meeting all inclusion criteria, is shown against the landscape of all gene sets in color, with each subsequent plot highlighting a specific subset of those gene sets. The vertical dashed red line highlights the $GOBP_{low}$ tPOD, the others highlight either the MSigDB $SUPER_{0.05}$ tPOD or the specific 5th percentile tPOD for the five individual supersets.

4.2.4 Proposed tPODs for *o*-Dichlorobenzene

As described above, EPA performed six distinct approaches to derive putative tPODs to be evaluated within the *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene* (U.S. EPA, 2026a), ranging from precautionary approaches consistent with the EPA Transcriptomic Assessment Product (ETAP; (U.S. EPA, 2024a, b)) towards a mechanism-informed approach utilizing a novel 5th percentile of the distribution of relevant "superset" approach (U.S. EPA, 2026e).

The six approaches are comprised of three collections of gene sets: (1) Gene Ontology Biological Process (GOBP) (The Gene Ontology Consortium, 2025; Ashburner et al., 2000), (2) Molecular

Signatures Database (MSigDB) (Castanza et al., 2023; Liberzon et al., 2015; Subramanian et al., 2005)), and (3) expert-curated, mechanism-informed superset terms specific for *o*-dichlorobenzene derived from the MSigDB collection. Within each of those three collections, 1) the lowest (*e.g.,* most sensitive) individual gene set that was modeled is shown as a precautionary metric, and 2) the 5th percentile of the distribution of all gene sets is shown as a more pertinent metric relating to exposure concentrations that would be anticipated to more closely mirror apical endpoints.

The $GOBP_{low}$ and $MSigDB_{low}$ gene set approaches provide a tPOD estimate that represents a dose below which coordinated transcriptional changes do not significantly differ from background and therefore do not indicate a toxicity of concern. These approaches do not consider mechanism, adversity, or known modes of action for observed toxic responses, and serve as the most sensitive indicators of potential biological activity. The tPODs generated from these approaches are sensitive measures of general *de minimis* biological effects that have retrospectively demonstrated good concordance with chronic, apical PODs [reviewed in (U.S. EPA, 2024a)]. They may work well for chemicals with no existing or publicly available repeat dose toxicity studies from which to inform mechanism or derive toxicity values using traditional risk assessment methods. On the other hand, a review of the existing data for *o*-dichlorobenzene identified adverse health outcomes in liver, lung, and kidney (Mörbt et al., 2011; ATSDR, 2006; U.S. EPA, 2006; Zissu, 1995; Hayes et al., 1985; NTP, 1985; Haskell Laboratories, 1982; Hollingsworth et al., 1958); therefore, EPA elected to develop an alternative approach to estimate tPODs. This "superset" approach used defined MSigDB gene sets related to apoptosis, necrosis, oxidative stress, cytotoxicity, and inflammation which are relevant to *o*-dichlorobenzene apical outcomes.

EPA derived tPODs based on both the lowest BMDL median value from the combined MSigDB supersets and the 5th percentile of the distribution of BMDL median values from the combined MSigDB supersets. The 5th percentile approach identified enrichment of gene sets plausibly associated with relevant toxicity outcomes that remained within approximately one order of magnitude of the inflection point of the accumulation plot curve, demonstrating that this percentile accurately reflected a POD estimate prior to maximal pathway activation which may act as a bioactivity threshold that is more representative of adversity compared to more precautionary approaches like those proposed in ETAP (U.S. EPA, 2024b). Additionally, the selection of this percentile aligns with established EPA risk assessment approaches (Dhond and Barron, 2022; Kroes et al., 2005; Newman et al., 2000; U.S. EPA, 1985) and has been used as the tPOD value within various *in vitro* NAMs and in the field of toxicogenomic research (Bundy et al., 2024; Harrill et al., 2024; Harrill et al., 2021; Friedman et al., 2020). Since traditional toxicity studies describing the apical effects of *o*-dichlorobenzene exposures were available, the EPA is proposing to use the 5th percentile BMDL median values from the combined MSigDB supersets in liver and lung as tPODs relevant for consideration in the *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene (*U.S. EPA, 2026a*)* as those values were more consistent with apical toxicity values; however, the $GOBP_{low}$ tPODs were also provided for comparison purposes (Table 4-7).

Ultimately, the combined superset $MSigDB_{0.05}$ tPODs for liver and lung (Table 4-7) are recommended for consideration as mechanism-informed tPODs for *o*-dichlorobenzene. These tPODs are being used as co-critical PODs in the evaluation of hazard for *o*-dichlorobenzene inhalation exposure, oral exposure, and skin contact (U.S. EPA, 2026a). Apical POD values can be found in Section 2.3 of the *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene* (U.S. EPA, 2026a).

Table 4-7. Proposed tPODs for Consideration in the *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene* (U.S. EPA, 2026a)

Species	Tissue	$GOBP_{low}$ tPOD (ppm)	$MSigDB_{0.05}$ Combined Superset tPOD (ppm)
Mouse	Liver	1.22	29.87
Mouse	Lung	1.21	6.67
Mouse	Kidney	1.01	4.72
Rat	Liver	41.1	112.49
Rat	Lung	205	218.79
Rat	Kidney	116	211.39

4.3 CAR Biomarker Gene Analysis

This section details the analysis EPA performed to identify whether short-term exposure to *p*-dichlorobenzene results in constitutive androstane receptor (CAR) activation, which is the proposed MIE for *p*-dichlorobenzene-induced liver tumors in mice. This rodent-specific MOA of liver tumorigenesis has been previously determined to not be relevant to human biology (U.S. EPA, 2026b). This CAR biomarker analysis was further used to investigate why *p*-dichlorobenzene exposure causes liver tumors in mice but not rats. CAR biomarker activity in response to *o*-dichlorobenzene exposure was also examined and is described in Appendix D.

4.3.1 Selection and Evaluation of CAR Biomarker Gene Set

The EPA *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene* (U.S. EPA, 2026b), which included assessment of adverse outcomes, mechanistic data, evidence integration, and weight of scientific evidence conclusions, identified a potential cancer hazard associated with increased rates of liver tumors in mice, but not rats. Although there was no evidence of genotoxicity in response to *p*-dichlorobenzene exposure, there was evidence for a nongenotoxic MOA including expression of biomarker genes and apical effects (*e.g.,* hepatocyte proliferation), providing some evidence for a CAR-mediated MOA underlying the mouse liver tumor response. Activation of CAR is a well-established, non-genotoxic mediator of rodent liver tumors (OECD, 2023) but not human liver tumors. A number of studies have been described which performed transcriptomic profiling of the livers of mice treated up to 90 days with *p*-dichlorobenzene (Henderson et al., 2015a; Eichner and Kossler, 2013; Thomas, 2009) which provided further evidence for the CAR MOA while potentially ruling out other MOAs.

There are several genes known to be differentially expressed upon CAR activation including some cytochrome P450s (CYPs) (Eichner et al., 2013). CYPs are a diverse superfamily of hemoproteins critical for Phase 1 metabolism across all domains of life. These enzymes catalyze the oxidative conversion of lipophilic compounds, including endogenous and exogenous substances, into more hydrophilic derivatives in preparation for excretion. CYPs are responsible for a majority of the oxidative metabolism of commonly used pharmaceuticals, essential endogenous compounds like steroid hormones and cholesterol, and environmental chemicals (as reviewed in (Pelkonen et al., 2008)). CYPs are highly conserved across eukaryotes due to their functional significance in steroidogenesis, lipid metabolism, and detoxification. There are highly conserved features between CYPs across eukaryotic organisms, most notably the heme-binding motif and a cysteine residue essential for oxygen activation, but there are differences in substrate specificity, gene expression, and regulatory mechanisms between species

including divergence between rodents and humans. The gene *Cyp2b10* in mice, is a highly inducible liver CYP that serves as the primary biomarker for activation of CAR (Elcombe et al., 2014). In humans, the functional analog for CYP2B10 is CYP2B6, and both mouse and human analogs are strongly induced by phenobarbital and other CAR agonists (Geter et al., 2014; Luisier et al., 2014). Cyp2c55, a CYP encoded by the gene *Cyp2c55* in mice, is predominantly expressed in colonic epithelium but also acts as a marker for hepatic CAR activation (Oshida et al., 2015). There is not a clear functional analog for *Cyp2c55* in humans, but members of the human CYP2C cluster are critical for drug metabolism and are induced by CAR. CDC20 is a remarkably conserved protein from yeast to humans with consistent functional capacity to activate the anaphase-promoting complex/cyclosome (APC/C) during mitosis. Because of its role in activating APC/C, CDC20 is often overexpressed in cancers and is activated by CAR (Negishi et al., 2020; Peffer et al., 2018; Tojima et al., 2012).

As a further line of evidence supporting CAR activation as the MIE driving downstream effects, the EPA identified a small biomarker set of genes known to be responsive to CAR agonists in mice (*Cdc20*, *Cyp2b10,* and *Cyp2c55*). Mechanistic studies have found that *p*-dichlorobenzene (and phenobarbital, a known CAR agonist) strongly activates *Cyp2b10* in mice ((Rooney et al., 2019; Pouché et al., 2017; Henderson et al., 2015b; Eichner et al., 2013; Tojima et al., 2012) and GSE68364 and GSE60684). Genes for *Cyp2b10* and *Cyp2c55* were among those upregulated in response to *p*-dichlorobenzene (and two other nongenotoxic liver carcinogens) (Eichner et al., 2013). Activation of *Cdc20* by CAR, due to it's role in cell proliferation, has been hypothesized to be involved in later key events including KE2 of sustained hepatocyte proliferation in the proposed CAR MOA in the *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene* (U.S. EPA, 2026b; Negishi et al., 2020; Peffer et al., 2018; Tojima et al., 2012).

The hepatic expression of these CAR biomarker genes in the 5-day *in vivo* transcriptomic studies for both *o*-dichlorobenzene and *p*-dichlorobenzene were examined to determine whether the observed transcriptional responses in liver are indicative of CAR activation, which would indicate the liver tumors were not human relevant. To compare transcriptional responses between species, mouse genes within the CAR biomarker gene set were mapped to their corresponding functionally orthologous genes in rat prior to analysis (mouse *Cdc20* is orthologous to rat *Cdc20*; mouse *Cyp2b10* is orthologous to rat *Cyp2b1* and *Cyp2b2*; mouse *Cyp2c55* is orthologous to rat *Cyp2c24*).

Exposure to *p*-dichlorobenzene resulted in large fold change increases across all genes in the CAR biomarker panel for both rat and mouse liver (Figure 4-23). For all genes, except *Cdc20*, the $\log_2$ fold change dose-response in mouse was left-shifted compared to rats at equivalent doses, indicating that mouse livers exposed to *p*-dichlorobenzene were more sensitive than rat, and the overall magnitude of change in expression in the CAR biomarker gene set in mice was more pronounced. The magnitude of fold change in expression of *Cdc20* in both mice and rats was not as robust as the *Cyp* genes. This may be due to *Cdc20*'s involvement in cell proliferation and more downstream KEs related to sustained hepatocellular proliferation, which the acute 5-day exposure in this transcriptomic study may not be able to detect. The BMD modeling results for the CAR biomarker gene set that met the criteria for modeling are presented in Table 4-8. Much like the responses in the $\log_2$ fold change profiles of the CAR biomarker genes, the modeled BMDs and BMDLs in mouse were much lower compared to rat indicating higher potency based on nominal exposure concentration.

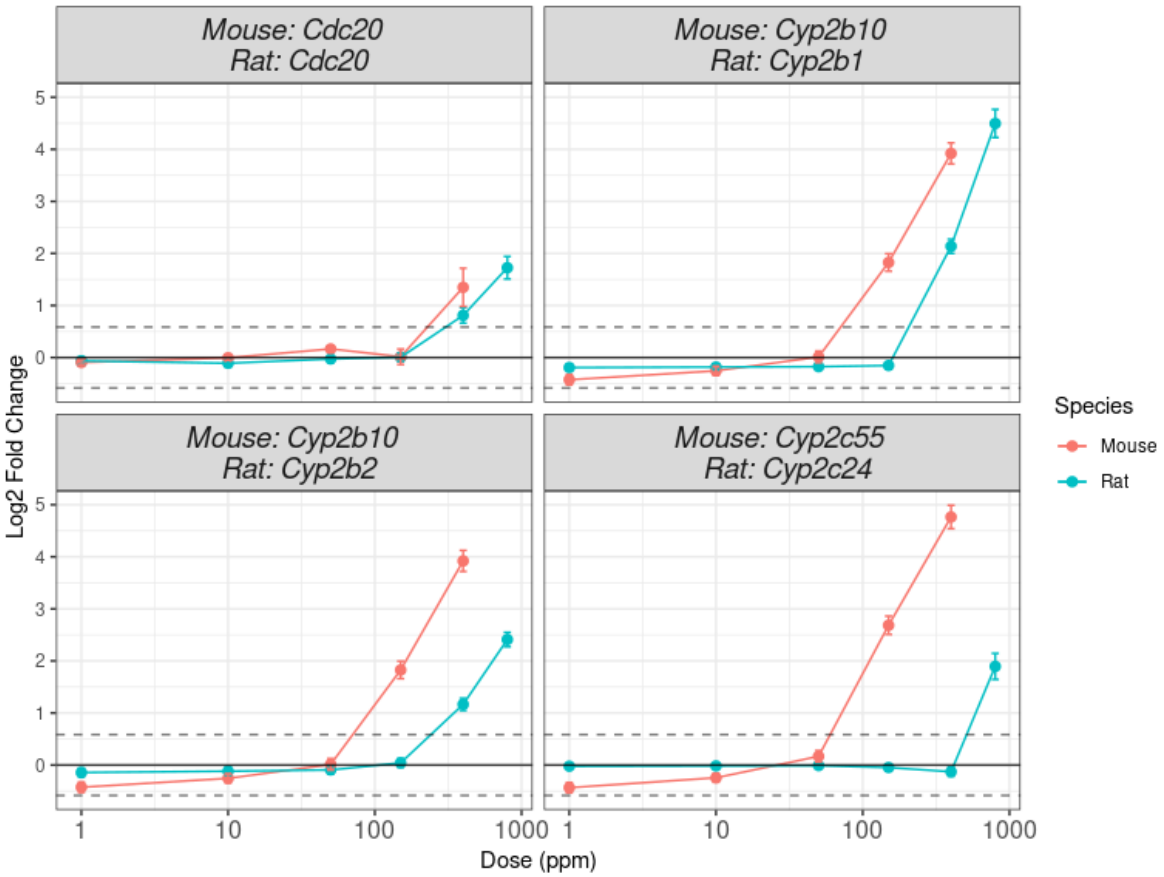

Figure 4-23. Dose-Response of the Log$_2$ Fold Change Expression of the CAR Biomarker Gene Set in Mouse and Rat Livers After *p*-Dichlorobenzene Exposure

Error bars denote the standard error in estimated log$_2$ fold change values. Horizontal dashed lines indicate an absolute log$_2$ fold change of 1.5 to designate a significant increase or decrease in fold change compared to vehicle control samples. Note, the log$_2$ fold change expression for Cyp2b1 and Cyp2b2 use the same data from the mouse Cyp2b10 ortholog.

Table 4-8. BMD Modeling Results of the CAR Biomarker Gene Set in Mouse or Rat Livers Exposed to *p*-Dichlorobenzene

Gene Symbol	Mouse BMD (ppm)	Mouse BMDL (ppm)	Rat BMD (ppm)	Rat BMDL (ppm)
Cdc20	58.33	13.41	253.48	200.92
Cyp2b1[a]	54.95	37.41	143.27	110.17
Cyp2b2[a]	54.95	37.41	190.69	131.65
Cyp2c24[b]	35.21	24.77	736.13	525.20

[a] Orthologous to mouse Cyp2b10
[b] Orthologous to mouse Cyp2c55
Note: Cyp2b1 and Cyp2b2 BMD modeling results for mouse are identical since they were calculated from the mouse ortholog, Cyp2b10

4.3.2 Species Comparison of CAR Biomarker Gene Set

As a result of the species differences observed in the magnitudes and potencies of the CAR biomarker gene set, differences in the overall hepatic response between mice and rats exposed to the same doses of

p-dichlorobenzene were examined to better quantify potential species differences. The highest comparable dose across both species was 400 ppm. *p*-dichlorobenzene exposure at 400 ppm in mice resulted in log_2 fold increases of 1.35 (*Cdc20*), 3.92 (*Cyp2b10*), and 4.76 (*Cyp2c55*). In contrast, the 400 ppm dose group in rat only elicited fold increases of 0.81 (*Cdc20*), 2.14 (*Cyp2b1*; functionally orthologous to mouse *Cyp2b10*), 1.17 (*Cyp2b2*; functionally orthologous to mouse *Cyp2b10*), and -0.13 (*Cyp2c24*; functionally orthologous to mouse *Cyp2c55*). Figure 4-24 examines the overall log_2 fold change difference between equivalent doses of *p*-dichlorobenzene in liver between mouse and rat. Rat *Cyp2c24* (functionally orthologous to mouse *Cyp2c55*) had the largest log_2 fold change difference at the highest equivalent dose of 400 ppm, with mouse having 4.89 times higher log_2 fold change expression compared to rat. Similarly, the log_2 expression of *Cyp2b1* and *Cypb2b2* (functionally orthologous to mouse *Cyp2b10*) was 1.78 and 2.76 times higher than rat at 400 ppm exposure in liver, respectively. *Cdc20* did not have a large expression difference between mouse and rat, with mouse only having 0.54 times higher log_2 expression compared to rat.

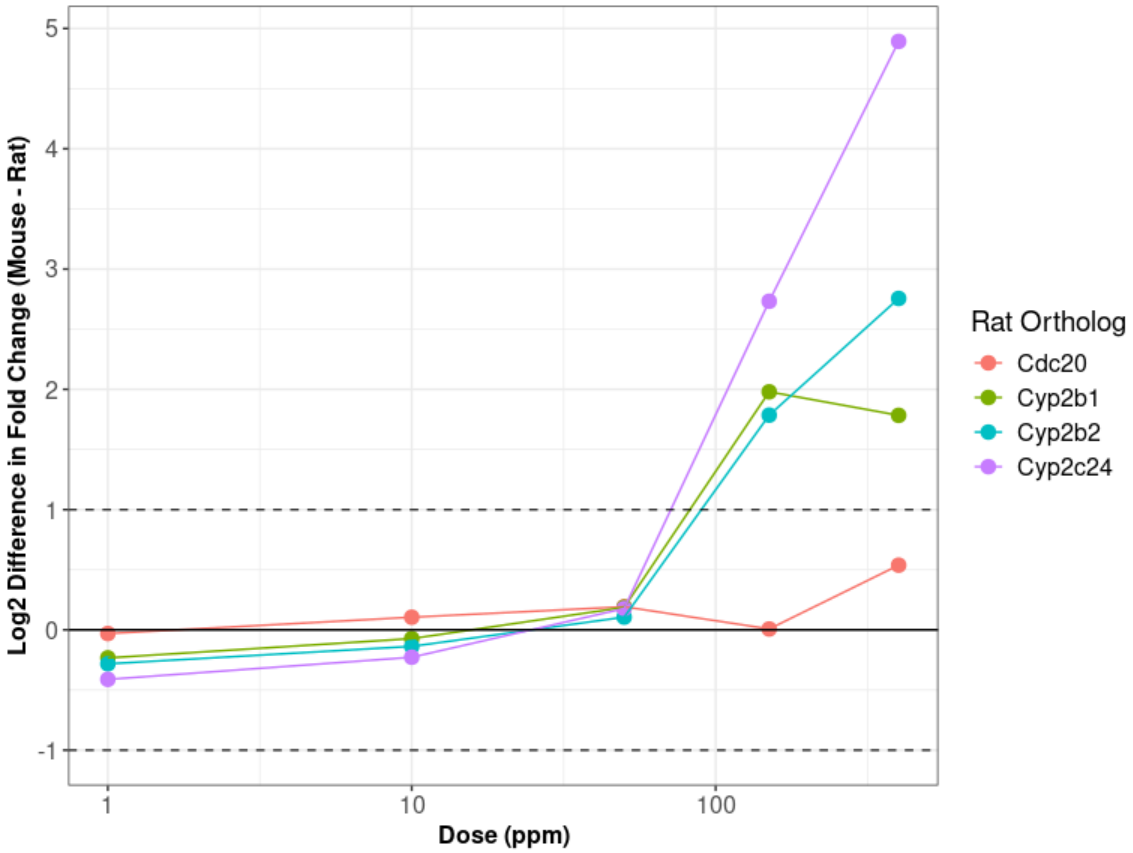

Figure 4-24. Species Comparison of Rat Orthologous CAR Biomarker Gene Set After *p*-Dichlorobenzene Exposure in Liver

Dashed horizontal lines indicate an absolute log_2 fold change difference between mouse and rat of 1 (arithmetic 2-fold increase or decrease compared to mouse). Note, the comparison of Cyp2b1 and Cyp2b2 use the same data from the mouse Cyp2b10 ortholog in the log_2 difference in fold change calculation.

5 SUMMARY AND CONCLUSIONS

In this draft TSD, EPA presents multiple analytical approaches to integrate existing knowledge from the *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene* (U.S. EPA, 2026a) and *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene* (U.S. EPA, 2026b) with the targeted transcriptomic data provided by the NIEHS 5-day *in vivo* studies. These analytical approaches were designed to (1) identify DEGs for each chemical, species, and tissue to assess the magnitude of response to chemical exposure across species, tissues, and dose levels, (2) perform dose-response modeling following the *Standard Methods for Development of EPA Transcriptomic Assessment Products* (U.S. EPA, 2024b) and subsequently analyze aggregated gene sets to determine tPODs, and (3) apply a targeted biomarker analysis to investigate the potential role of CAR in *p*-dichlorobenzene and *o*-dichlorobenzene hazard evaluations. Furthermore, a variety of approaches were utilized in the analyses for *o*-dichlorobenzene and *p*-dichlorobenzene to evaluate the appropriateness of mechanism-informed tPOD derivation.

5.1 *o*-Dichlorobenzene Summary

EPA's analysis of adverse outcomes, mechanistic data, evidence integration and weight of scientific evidence conclusions for the most relevant human health hazard outcomes for *o*-dichlorobenzene identified well-supported non-cancer hazards associated with liver, respiratory system, and kidney toxicity. This evidence informed the approaches for transcriptomic analysis of liver, lung, and kidney tissue (with heart and ovary tissue included as comparators) from the NIEHS 5-day inhalation study in female mice and rats (NIEHS, 2025b). Synthesis of the available mechanistic and histopathological effects associated with *o*-dichlorobenzene exposure across these tissues suggested apoptosis, necrosis, oxidative stress, cytotoxicity, and inflammatory processes as cellular processes/KEs plausibly relevant to *o*-dichlorobenzene toxicity (U.S. EPA, 2026a). To evaluate whether transcriptomic data supported the involvement of these KEs in *o*-dichlorobenzene toxicity for each tissue, and to provide a mechanism-informed tPOD for *o*-dichlorobenzene, a novel BMD analysis was performed. This analysis aggregated MSigDB gene sets involved in apoptosis, necrosis, oxidative stress, cytotoxicity, and inflammation into "supersets" prior to BMD analysis and tPOD identification. It is important to note that MSigDB gene sets, which are often experimentally and computationally linked to adverse effects at the cellular level, may not always translate to specific adverse effects at the apical level. However, the existing mechanistic and apical data associated with *o*-dichlorobenzene, effectively informed gene set aggregation (supersets) and served as a bridge between early molecular changes and the later observed adverse apical effects. Whether this approach can be applied to other chemicals may require further study.

The MsigDB tPOD of each superset and the combined superset for each tissue was integrated into the weight of evidence analysis of *o*-dichlorobenzene. As the MSigDB gene sets for mouse were comprised of mouse-native gene sets, rat gene required orthology mapping from human gene sets because there are no rat native gene sets available in MSigDB. BMD modeling was performed across genes mapped to individual gene set categories and in aggregate to identify the gene set-specific $MSigDB_{low}$ tPODs and $MSigDB_{0.05}$ tPODs for liver, lung, and kidney tissues in both mice and rats. The $GOBP_{low}$ and $GOBP_{0.05}$ tPODs were also identified for comparison purposes. The *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene* (U.S. EPA, 2026a) utilized the 5th percentile of the distribution of MSigDB BMDL median values for superset terms related to apoptosis, cytotoxicity, inflammation, oxidative stress, and necrosis.. The combined superset $MSigDB_{0.05}$ tPODs (liver: 29.87 ppm and lung: 6.67 ppm) are recommended for consideration as mechanism-informed tPODs for *o*-dichlorobenzene. These tPODs serve as representative markers for the level of *o*-dichlorobenzene that could cause short-term liver and lung harm and are being used as co-critical PODs in the evaluation of

hazard for *o*-dichlorobenzene inhalation exposure, oral exposure, and skin contact (U.S. EPA, 2026a). Although apical studies have not demonstrated evidence that the proposed MOA for *p*-dichlorobenzene-induced liver tumors is also relevant for *o*-dichlorobenzene, enrichment of the CAR biomarker gene set was investigated following *o*-dichlorobenzene exposure to characterize CAR-specific activity and facilitate comparison of potencies across dichlorobenzene isomers in mouse and rat liver (see Appendix D).

5.2 *p*-Dichlorobenzene Summary

Analysis of adverse outcomes, mechanistic data, evidence integration, and weight of scientific evidence conclusions for the most relevant human health hazard outcomes for *p*-dichlorobenzene identified potential cancer hazard associated with liver (U.S. EPA, 2026b). Exposure to *p*-dichlorobenzene resulted in dose dependent liver tumors in mice but not rats. There was no evidence of genotoxicity and no additional mechanistic data to evaluate non-genotoxic modes of action which could establish human health relevance. Previously, short-term mechanistic studies implicated CAR activation following *p*-dichlorobenzene exposure (Henderson et al., 2015a; Eichner and Kossler, 2013; Thomas, 2009). Activation of CAR is a well-established, non-genotoxic mediator of rodent-specific liver tumors (OECD, 2023) and, therefore, not relevant to the process of human liver tumorigenesis.

To further investigate mechanisms of *p*-dichlorobenzene toxicity and compare the effects of *o*-dichlorobenzene with *p*-dichlorobenzene, the MSigDB superset tPOD analysis using the same terms determined for *o*-dichlorobenzene (apoptosis, cytotoxicity, inflammation, oxidative stress, necrosis) was applied to *p*-dichlorobenzene. The *o*-dichlorobenzene terms provided poor coverage for *p*-dichlorobenzene effects, so an additional superset list was custom-curated based on the transcriptomic data and overlap with the *p*-dichlorobenzene hazard document. Identified terms were related to inflammation, cell cycle, lipid metabolism, oxidative stress, and cell transport and provided potentially more relevant tPODs in response to *p*-dichlorobenzene exposure compared to approaches not based on supersets. This additional analysis is presented in Appendix C. EPA has determined that the tPODs from this analysis are not appropriate to consider for risk assessment of *p*-dichlorobenzene. Analysis of the tPOD results indicates that *o*-dichlorobenzene specific superset values are not very sensitive to *p*-dichlorobenzene specific apical effects. Alternate analyses using curated superset terms for *p*-dichlorobenzene were more appropriate and closer to the apical values. Additionally, as discussed in Section 2.3.1 of the *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene* (U.S. EPA, 2026b), the hazard database for *p*-dichlorobenzene is thorough and robust, with a complete set of Federal Insecticide, Fungicide, and Rodenticide Act toxicity studies available in addition to other studies identified through systematic review (see Section 2.2.1 of the *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene* (U.S. EPA, 2026b)). For these reasons, use of the tPODs summarized in Appendix C was not considered in the *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene* (U.S. EPA, 2026b).

To better understand the mechanism of *p*-dichlorobenzene liver tumorigenesis and species differences between mice and rats, EPA performed a transcriptomic analysis in mouse and rat livers with a focus on previously established CAR biomarker genes. This analysis was proposed for use in the *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene* (U.S. EPA, 2026b) to determine whether CAR induction is likely responsible for the observed incidence of rodent liver tumors following exposure to *p*-dichlorobenzene. Results demonstrated strong hepatic induction of the CAR biomarker genes after exposure to *p*-dichlorobenzene in mouse and rat. Comparison of hepatic induction of these CAR genes between mouse and rat demonstrated that mouse livers had much larger induction of these genes, suggesting that CAR activity may be particularly relevant in mice. This difference in the

magnitude of CAR-mediated gene expression may explain why *p*-dichlorobenzene exposure results in liver tumors in mice, but not in rats (Aiso et al., 2005; JBRC, 1995; NTP, 1987). Additional, exploratory analysis involving mapping of coordinated changes in gene expression to annotated gene set databases further informed the mechanisms underlying *p*-dichlorobenzene-mediated tumorigenesis. Collectively, EPA is proposing this analysis for use in the *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene* (U.S. EPA, 2026b) by providing transcriptomic evidence that *p*-dichlorobenzene activates CAR and is the biologically plausible MIE for the proposed MOA of CAR-mediated mouse liver tumorigenesis.

5.3 Limitations

An important consideration across all reported results is that all data were modeled following the reported nominal (exposure) concentrations (U.S. EPA, 2024b); differences in toxicokinetics could result in tissue- or species-specific differences that were not accounted for in the tPODs described. Estimated inhaled doses as well as terminal concentrations of *o*-dichlorobenzene and *p*-dichlorobenzene in blood, liver, and lung can be found in the full NIEHS reports (NIEHS, 2025a, b).

Additionally, use of distribution-based approaches, such as the 5th percentile of the distribution of gene set BMDL median values, for deriving tPODs can be subject to inherent variability across tissues and experiments due to differences in the extent of transcriptional change induced by a particular chemical (*i.e.,* number of gene sets where at least three genes were annotated and had a BMD). Nevertheless, the 5th percentile approach targets the most responsive genes that may contribute to the toxicological response. Furthermore, EPA demonstrated that for this study, the 5th percentile of the distribution of gene set BMDL median values approach utilized for the *o*-dichlorobenzene transcriptomic data identified enrichment of hazard assessment-informed gene sets plausibly associated with relevant toxicity outcomes while remaining within approximately one order of magnitude of the inflection point of the BMDL median accumulation plot curve.

Another important consideration when interpreting these results is that the transcriptomic analysis measured a subset of 2,756 mouse and 2,602 rat genes in contrast to the whole transcriptome. This subset was designed to maximize biological pathway coverage while minimizing sequencing cost (Mav et al., 2018), which may limit its utility in investigating chemical specific MOAs and biomarker analyses as certain genes may not be represented in the panel.

Finally, transcriptomics is measuring mRNA expression at a snapshot in time. Transcriptomic changes are not inherently adverse; rather, changes in gene expression can be necessary for normal function, adaptive in response to a stressor, neutral if they do not result in changes at the protein level, or adverse. However, the presented analyses attempt to mitigate this limitation by proposing an analysis that is mechanism-informed and anchored to the phenotypic outcomes evaluated in the *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene* (U.S. EPA, 2026a) and the *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene* (U.S. EPA, 2026b).

5.4 Conclusions

In support of the EPA's mission to protect human health and the environment, the Agency performs human health risk assessments to estimate the nature and probability of adverse health effects in humans exposed to chemicals. Although these assessments historically have relied on traditional chronic animal bioassays that have a long history of use in risk characterization and management, regulatory agencies including the EPA are aiming to transition away from the use of mammalian animal testing. In accordance with the TSCA amendment of the Frank R. Lautenberg Chemical Safety for the 21st Century

Act on June 22, 2016, and TSCA section 4(h)(1)(B), the EPA recommitted on January 22, 2026, to phasing out mammalian animal testing and further incorporating NAMs into chemical risk evaluations.

This draft TSD for two chemicals, *o*-dichlorobenzene and *p*-dichlorobenzene, serves to further this commitment by presenting the first incorporation of transcriptomic data substantively into a forthcoming formal risk evaluation. The 5-day transcriptomic paradigm, although using animals, serves as a necessary refinement bridge to validate approaches with better ethical and financial perspectives while improving MOA understanding and filling data gaps with the eventual goal to replace traditional chronic animal bioassays. NIEHS conducted 5-day targeted transcriptomic studies for *o*-dichlorobenzene and *p*-dichlorobenzene by exposing mice and rats to inhaled chemicals for use in forthcoming TSCA draft risk evaluations, with the full methods for this study described by NIEHS DTT (NIEHS, 2025a, b). Using existing evidence from studies that examined the apical effects of exposure allowed the EPA to develop a novel analysis that provides powerful lines of evidence for relating exposure to the proposed health effects for these chemicals. The combined superset $MSigDB_{0.05}$ tPODs (liver: 29.87 ppm and lung: 6.67 ppm) are recommended for consideration as mechanism-informed tPODs for *o*-dichlorobenzene. These tPODs serve as representative markers for the level of *o*-dichlorobenzene that could cause short-term liver and lung harm and are being used as co-critical PODs in the evaluation of hazard for *o*-dichlorobenzene inhalation exposure, oral exposure, and skin contact (U.S. EPA, 2026a).

This draft TSD is being released for public comment and peer review by the independent SACC in June 2026. EPA is soliciting input on topics associated with experimental design, analysis methodology, soundness of conclusions, and use of the results to support the *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene* (U.S. EPA, 2026a) and *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene* (U.S. EPA, 2026b)

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APPENDICES

Appendix A SUPPORTING MATERIALS AND METHODS

A.1 Overview

Complete methods for the *o*-dichlorobenzene and *p*-dichlorobenzene 5-day *in vivo* transcriptomic studies performed by NIEHS DTT, including chemical procurement, study design, chemical exposure, dose formulation, sample collection, clinical examinations, and internal concentration assessments, are available (NIEHS, 2025a, b). In brief, female SD rats and female B6D2F1/Crl mice were exposed via whole-body inhalation 6 hours per day for 5 consecutive days to five doses of *o*-dichlorobenzene or *p*-dichlorobenzene and a vehicle control in mice or six doses of *o*-dichlorobenzene or *p*-dichlorobenzene and a vehicle control in rats. At the end of the exposure period, gene expression changes were measured in the heart, kidney, liver, lung, and ovary.

A.2 Sequence Alignment, Sample Normalization, and Quality Control

Raw sequencing data (FASTQ) files of Templated Oligo with Sequencing Readout (TempO-Seq) reads from the 5-day *in vivo* transcriptomic studies were downloaded from the National Center for Biotechnology Information Sequence Read Archive database under BioProject accession numbers PRJNA1304578 (rat *o*-dichlorobenzene and *p*-dichlorobenzene study) and PRJNA1304935 (mouse *o*-dichlorobenzene and *p*-dichlorobenzene study). Data delivery of the original 759 FASTQ files and corresponding metadata was requested via the Sequence Read Archive Cloud Data Delivery service on October 02, 2025, and the files were delivered to an EPA-hosted Amazon Simple Storage Service bucket on October 04, 2025, for access.

The sequence alignment and sample normalization performed by EPA followed the process outlined in the *Standard Methods for Development of EPA Transcriptomic Assessment Products* (U.S. EPA, 2024b). Briefly, each FASTQ file was aligned to the TempO-Seq probe manifest using HISAT2 and imported directly into SAMtools to compute probe-level counts for each individual FASTQ file. Samples were evaluated for quality based on the following:

- Any technical issues identified during sample collection, preparation, or sequencing were removed from further analysis.
- Sequencing depth (*i.e.,* total number of mapped reads). Samples with less than 10% of the approximate median sequencing depth calculated across all samples prior to applying filtering were removed from further analysis.
    - For mouse, the approximate median sequencing depth was 9,000,000 mapped reads (actual median = 9,251,432). The minimum sequencing depth cutoff was set to 900,000 mapped reads.
    - For rats, the approximate median sequencing depth was 8,000,000 mapped reads (actual median = 8,256,231). The minimum sequencing depth cutoff was set to 800,000 mapped reads.
- Fraction of uniquely mapped reads. Samples with less than 50% of reads uniquely mapped to known probes were removed from further analysis.
- Probe coverage (*i.e.,* total probes with at least 5 reads). Samples with less than 1,200 covered probes were removed from further analysis.

The lists of samples removed based on the sequence alignment quality control (QC) metrics are listed in Table_Apx A-1 and Table_Apx A-2. It should be noted that 24.3% (9/37) and 20% (7/35) of all mouse kidney samples for *o*-dichlorobenzene and *p*-dichlorobenzene, respectively, had evidence of tissue contamination and were removed, resulting in lower sample sizes for kidney than other tissues. Specifically, NIEHS DTT identified a subset of samples via distinct PCA clustering as potential outliers; this separation was driven by very high expression of pancreas-specific genes compared to other kidney samples, which suggests possible contamination with pancreatic tissue during sample collection.

Table_Apx A-1. Samples Removed Based on QC Metrics in Rats

Tissue	Animal ID	Chemical	Dose Group	QC Issue
Kidney	2DR1F1	*o*-Dichlorobenzene	Vehicle	<50% reads aligned
Lung	2DR7F601	*o*-Dichlorobenzene	500 ppm	<50% reads aligned
Lung	4DR1F2	*p*-Dichlorobenzene	Vehicle	<50% reads aligned
Lung	4DR1F8	*p*-Dichlorobenzene	Vehicle	<50% reads aligned
Lung	4DR2F102	*p*-Ddichlorobenzene	1 ppm	<50% reads aligned
Lung	4DR3F204	*p*-Dichlorobenzene	10 ppm	<50% reads aligned
Lung	4DR5F402	*p*-Ddichlorobenzene	150 ppm	<50% reads aligned
Ovary	4DR1F6	*p*-Ddichlorobenzene	Vehicle	<10% target depth

Table_Apx A-2. Samples Removed Based on QC Metrics in Mice

Tissue	Animal ID	Chemical	Dose Group	QC Issue
Kidney	2DM2F105	*o*-dichlorobenzene	1 ppm	<50% reads aligned
Kidney	2DM1F1	*o*-dichlorobenzene	Vehicle	Tissue contamination
Kidney	2DM1F4	*o*-dichlorobenzene	Vehicle	Tissue contamination
Kidney	2DM1F6	*o*-dichlorobenzene	Vehicle	Tissue contamination
Kidney	2DM1F9	*o*-dichlorobenzene	Vehicle	Tissue contamination
Kidney	2DM3F202	*o*-dichlorobenzene	10 ppm	Tissue contamination
Kidney	2DM4F303	*o*-dichlorobenzene	30 ppm	Tissue contamination
Kidney	2DM5F401	*o*-dichlorobenzene	100 ppm	Tissue contamination
Kidney	2DM5F402	*o*-dichlorobenzene	100 ppm	Tissue contamination
Kidney	2DM6F501	*o*-dichlorobenzene	250 ppm	Tissue contamination
Liver	2DM1F9	*o*-dichlorobenzene	Vehicle	<50% reads aligned
Liver	2DM2F101	*o*-dichlorobenzene	1 ppm	<50% reads aligned
Liver	2DM2F102	*o*-dichlorobenzene	1 ppm	<50% reads aligned
Liver	2DM3F203	*o*-dichlorobenzene	10 ppm	<50% reads aligned
Liver	2DM6F504	*o*-dichlorobenzene	250 ppm	<50% reads aligned
Lung	2DM6F505	*o*-dichlorobenzene	250 ppm	<50% reads aligned
Kidney	4DM1F9	*p*-dichlorobenzene	Vehicle	<10% target depth
Kidney	4DM1F1	*p*-dichlorobenzene	Vehicle	Tissue contamination
Kidney	4DM1F4	*p*-dichlorobenzene	Vehicle	Tissue contamination
Kidney	4DM1F5	*p*-dichlorobenzene	Vehicle	Tissue contamination
Kidney	4DM2F105	*p*-dichlorobenzene	1 ppm	Tissue contamination
Kidney	4DM3F202	*p*-dichlorobenzene	10 ppm	Tissue contamination
Kidney	4DM4F303	*p*-dichlorobenzene	50 ppm	Tissue contamination
Kidney	4DM5F402	*p*-dichlorobenzene	150 ppm	Tissue contamination
Liver	4DM1F6	*p*-dichlorobenzene	Vehicle	<50% reads aligned
Liver	4DM1F8	*p*-dichlorobenzene	Vehicle	<50% reads aligned
Liver	4DM1F9	*p*-dichlorobenzene	Vehicle	<50% reads aligned
Liver	4DM3F204	*p*-dichlorobenzene	10 ppm	<50% reads aligned

Prior to performing BMD modeling with BMDExpress, probe counts for each sample were normalized to adjust for differences in sequencing depth. For each chemical treatment condition in each species and tissue, raw probe counts for all samples were normalized within each sample as follows:

- All probes with a mean read count less than 5 were removed, as these probes lack sufficient signal for reliable analysis.
- Each remaining probe count was normalized to counts per million (CPM = probe count × 1,000,000 ÷ sum of all remaining probe counts in sample)
- CPM values transformed to $\log_2$ scale with added pseudo-count of 1 to prevent taking log of zero counts and ensuring a positive value for dose-response modeling.

To identify potential outlier samples, a principal component analysis (PCA) was performed on subsets of samples for both rats and mice corresponding to either: (1) all samples corresponding to same tissue, including matched vehicle controls ("treatment PCA"); and (2) all available vehicle controls corresponding to the same tissue ("vehicle PCA"). Samples not meeting the sequencing quality metrics

(*e.g.,* <50% of uniquely aligned reads) were excluded prior to PCA analysis. Outlier samples were identified based on the following considerations:

- Individual samples separated from all remaining samples on either principal component #1 (PC1) or principal component #2 (PC2) by greater than 2× the span of all other samples on the corresponding PC were considered strong outliers and removed from further analysis.
- Individual samples separated by less than 2× the range of all other samples were considered moderate outliers, and additional exclusion criteria were considered:
    - Vehicle samples that appeared as moderate outliers on both a treatment PCA and vehicle PCA were excluded unless multiple controls from the same group appeared as outliers.
    - Moderate outlier samples with lower quality than corresponding tissue samples by one or more sequencing quality metrics (*e.g.,* percentage of uniquely mapped reads) were excluded.
    - Samples that appeared as moderate outliers in both PC1 and PC2 with a relatively large Euclidean distance from all other remaining samples were excluded.
    - Moderate outlier samples that were especially distant from corresponding replicates or similar doses were excluded.

When multiple outlier samples were present on the same PCA, they were only removed if each outlier sample corresponded to a different dose group, as these were unlikely to represent any reproducible dose-dependent effect. The outlier samples that were removed in the rat and mouse *o*-dichlorobenzene and *p*-dichlorobenzene studies are listed in Table_Apx A-3 and Table_Apx A-4, respectively. The National Center for Biotechnology Information Sequence Read Archive accession numbers for these datasets are: PRJNA1304578 (rat) and PRJNA1304935 (mouse).

Table_Apx A-3. Samples Removed Based on PCA Grouped by Tissue and Chemical in Rat

Tissue	Animal ID	Chemical	Dose Group
Kidney	4DR1F1	*p*-Dichlorobenzene	Vehicle

Table_Apx A-4. Samples Removed Based on PCA Grouped by Tissue and Chemical in Mouse

Tissue	Animal ID	Chemical	Dose Group
Kidney	2DM1F5	*o*-Dichlorobenzene	Vehicle
Kidney	2DM4F302	*o*-Dichlorobenzene	30 ppm
Liver	2DM2F105	*o*-Dichlorobenzene	1 ppm
Lung	2DM2F104	*o*-Dichlorobenzene	1 ppm
Heart	4DM5F405	*p*-Dichlorobenzene	150 ppm
Kidney	4DM1F2	*p*-Dichlorobenzene	Vehicle
Liver	4DM5F403	*p*-Dichlorobenzene	150 ppm

The final sample sizes for all dose groups after outlier removal in rat and mouse *p*-dichlorobenzene and *o*-dichlorobenzene studies are summarized in Table_Apx A-5 and Table_Apx A-6, respectively. As noted earlier, many kidney tissue samples in mice had evidence of tissue contamination and were removed from analysis. However, the number of remaining samples across all dose groups and vehicle control groups for both *o*-dichlorobenzene and *p*-dichlorobenzene was above the minimum number of samples required to pass quality control and outlier detection to proceed with dose-response modeling

(N = 3 for vehicle control groups and N = 2 for each dose group) following the EPA Transcriptomics Assessment Product Standard Methods (U.S. EPA, 2024b). Samples removed due to quality issues were excluded from all subsequent BMD modeling and DESeq2 differential expression analyses.

Table_Apx A-5. Final Sample Sizes by Tissue, Chemical, and Dose Group in Rat

Tissue	Chemical	Dose Group	Sample Size
Heart	*o*-Dichlorobenzene	Vehicle	10
Heart	*o*-Dichlorobenzene	1 ppm	5
Heart	*o*-Dichlorobenzene	10 ppm	5
Heart	*o*-Dichlorobenzene	30 ppm	5
Heart	*o*-Dichlorobenzene	100 ppm	5
Heart	*o*-Dichlorobenzene	250 ppm	5
Heart	*o*-Dichlorobenzene	500 ppm	5
Kidney	*o*-Dichlorobenzene	Vehicle	9
Kidney	*o*-Dichlorobenzene	1 ppm	5
Kidney	*o*-Dichlorobenzene	10 ppm	5
Kidney	*o*-Dichlorobenzene	30 ppm	5
Kidney	*o*-Dichlorobenzene	100 ppm	5
Kidney	*o*-Dichlorobenzene	250 ppm	5
Kidney	*o*-Dichlorobenzene	500 ppm	5
Liver	*o*-Dichlorobenzene	Vehicle	10
Liver	*o*-Dichlorobenzene	1 ppm	5
Liver	*o*-Dichlorobenzene	10 ppm	5
Liver	*o*-Dichlorobenzene	30 ppm	5
Liver	*o*-Dichlorobenzene	100 ppm	5
Liver	*o*-Dichlorobenzene	250 ppm	5
Liver	*o*-Dichlorobenzene	500 ppm	5
Lung	*o*-Dichlorobenzene	Vehicle	10
Lung	*o*-Dichlorobenzene	1 ppm	5
Lung	*o*-Dichlorobenzene	10 ppm	5
Lung	*o*-Dichlorobenzene	30 ppm	5
Lung	*o*-Dichlorobenzene	100 ppm	5
Lung	*o*-Dichlorobenzene	250 ppm	5
Lung	*o*-Dichlorobenzene	500 ppm	4
Ovary	*o*-Dichlorobenzene	Vehicle	10
Ovary	*o*-Dichlorobenzene	1 ppm	5
Ovary	*o*-Dichlorobenzene	10 ppm	5
Ovary	*o*-Dichlorobenzene	30 ppm	5
Ovary	*o*-Dichlorobenzene	100 ppm	5
Ovary	*o*-Dichlorobenzene	250 ppm	5
Ovary	*o*-Dichlorobenzene	500 ppm	5
Heart	*p*-Dichlorobenzene	Vehicle	10
Heart	*p*-Dichlorobenzene	1 ppm	5
Heart	*p*-Dichlorobenzene	10 ppm	5
Heart	*p*-Dichlorobenzene	50 ppm	5
Heart	*p*-Dichlorobenzene	150 ppm	5
Heart	*p*-Dichlorobenzene	400 ppm	5
Heart	*p*-Dichlorobenzene	800 ppm	5
Kidney	*p*-Dichlorobenzene	Vehicle	9
Kidney	*p*-Dichlorobenzene	1 ppm	4

Tissue	Chemical	Dose Group	Sample Size
Kidney	*p*-Dichlorobenzene	10 ppm	5
Kidney	*p*-Dichlorobenzene	50 ppm	5
Kidney	*p*-Dichlorobenzene	150 ppm	5
Kidney	*p*-Dichlorobenzene	400 ppm	5
Kidney	*p*-Dichlorobenzene	800 ppm	5
Liver	*p*-Dichlorobenzene	Vehicle	10
Liver	*p*-Dichlorobenzene	1 ppm	5
Liver	*p*-Dichlorobenzene	10 ppm	5
Liver	*p*-Dichlorobenzene	50 ppm	5
Liver	*p*-Dichlorobenzene	150 ppm	5
Liver	*p*-Dichlorobenzene	400 ppm	5
Liver	*p*-Dichlorobenzene	800 ppm	5
Lung	*p*-Dichlorobenzene	Vehicle	8
Lung	*p*-Dichlorobenzene	1 ppm	4
Lung	*p*-Dichlorobenzene	10 ppm	4
Lung	*p*-Dichlorobenzene	50 ppm	5
Lung	*p*-Dichlorobenzene	150 ppm	4
Lung	*p*-Dichlorobenzene	400 ppm	5
Lung	*p*-Dichlorobenzene	800 ppm	5
Ovary	*p*-Dichlorobenzene	Vehicle	9
Ovary	p-Dichlorobenzene	1 ppm	5
Ovary	*p*-Dichlorobenzene	10 ppm	5
Ovary	*p*-Dichlorobenzene	50 ppm	5
Ovary	*p*-Dichlorobenzene	150 ppm	5
Ovary	*p*-Dichlorobenzene	400 ppm	5
Ovary	*p*-Dichlorobenzene	800 ppm	5

Table_Apx A-6. Final Sample Sizes by Tissue, Chemical, and Dose Group in Mouse

Tissue	Chemical	Dose Group	Sample Size
Heart	*o*-Dichlorobenzene	Vehicle	10
Heart	*o*-Dichlorobenzene	1 ppm	5
Heart	*o*-Dichlorobenzene	10 ppm	5
Heart	*o*-Dichlorobenzene	30 ppm	5
Heart	*o*-Dichlorobenzene	100 ppm	5
Heart	*o*-Dichlorobenzene	250 ppm	7
Kidney	*o*-Dichlorobenzene	Vehicle	5
Kidney	*o*-Dichlorobenzene	1 ppm	4
Kidney	*o*-Dichlorobenzene	10 ppm	4
Kidney	*o*-Dichlorobenzene	30 ppm	3
Kidney	*o*-Dichlorobenzene	100 ppm	3
Kidney	*o*-Dichlorobenzene	250 ppm	6
Liver	*o*-Dichlorobenzene	Vehicle	9
Liver	*o*-Dichlorobenzene	1 ppm	2
Liver	*o*-Dichlorobenzene	10 ppm	4
Liver	*o*-Dichlorobenzene	30 ppm	5
Liver	*o*-Dichlorobenzene	100 ppm	5
Liver	*o*-Dichlorobenzene	250 ppm	6
Lung	*o*-Dichlorobenzene	Vehicle	10

Tissue	Chemical	Dose Group	Sample Size
Lung	*o*-Dichlorobenzene	1 ppm	4
Lung	*o*-Dichlorobenzene	10 ppm	5
Lung	*o*-Dichlorobenzene	30 ppm	5
Lung	*o*-Dichlorobenzene	100 ppm	5
Lung	*o*-Dichlorobenzene	250 ppm	6
Ovary	*o*-Dichlorobenzene	Vehicle	10
Ovary	*o*-Dichlorobenzene	1 ppm	5
Ovary	*o*-Dichlorobenzene	10 ppm	5
Ovary	*o*-Dichlorobenzene	30 ppm	5
Ovary	*o*-Dichlorobenzene	100 ppm	5
Ovary	*o*-Dichlorobenzene	250 ppm	7
Heart	*p*-Dichlorobenzene	Vehicle	10
Heart	*p*-Dichlorobenzene	1 ppm	5
Heart	*p*-Dichlorobenzene	10 ppm	5
Heart	*p*-Dichlorobenzene	50 ppm	5
Heart	*p*-Dichlorobenzene	150 ppm	4
Heart	*p*-Dichlorobenzene	400 ppm	5
Kidney	*p*-Dichlorobenzene	Vehicle	5
Kidney	*p*-Dichlorobenzene	1 ppm	4
Kidney	*p*-Dichlorobenzene	10 ppm	4
Kidney	*p*-Dichlorobenzene	50 ppm	4
Kidney	*p*-Dichlorobenzene	150 ppm	4
Kidney	*p*-Dichlorobenzene	400 ppm	5
Liver	*p*-Dichlorobenzene	Vehicle	7
Liver	*p*-Dichlorobenzene	1 ppm	5
Liver	*p*-Dichlorobenzene	10 ppm	4
Liver	*p*-Dichlorobenzene	50 ppm	5
Liver	*p*-Dichlorobenzene	150 ppm	4
Liver	*p*-Dichlorobenzene	400 ppm	5
Lung	*p*-Dichlorobenzene	Vehicle	10
Lung	*p*-Dichlorobenzene	1 ppm	5
Lung	*p*-Dichlorobenzene	10 ppm	5
Lung	*p*-Dichlorobenzene	50 ppm	5
Lung	*p*-Dichlorobenzene	150 ppm	5
Lung	*p*-Dichlorobenzene	400 ppm	5
Ovary	*p*-Dichlorobenzene	Vehicle	10
Ovary	*p*-Dichlorobenzene	1 ppm	5
Ovary	*p*-Dichlorobenzene	10 ppm	5
Ovary	*p*-Dichlorobenzene	50 ppm	5
Ovary	*p*-Dichlorobenzene	150 ppm	5
Ovary	*p*-Dichlorobenzene	400 ppm	5

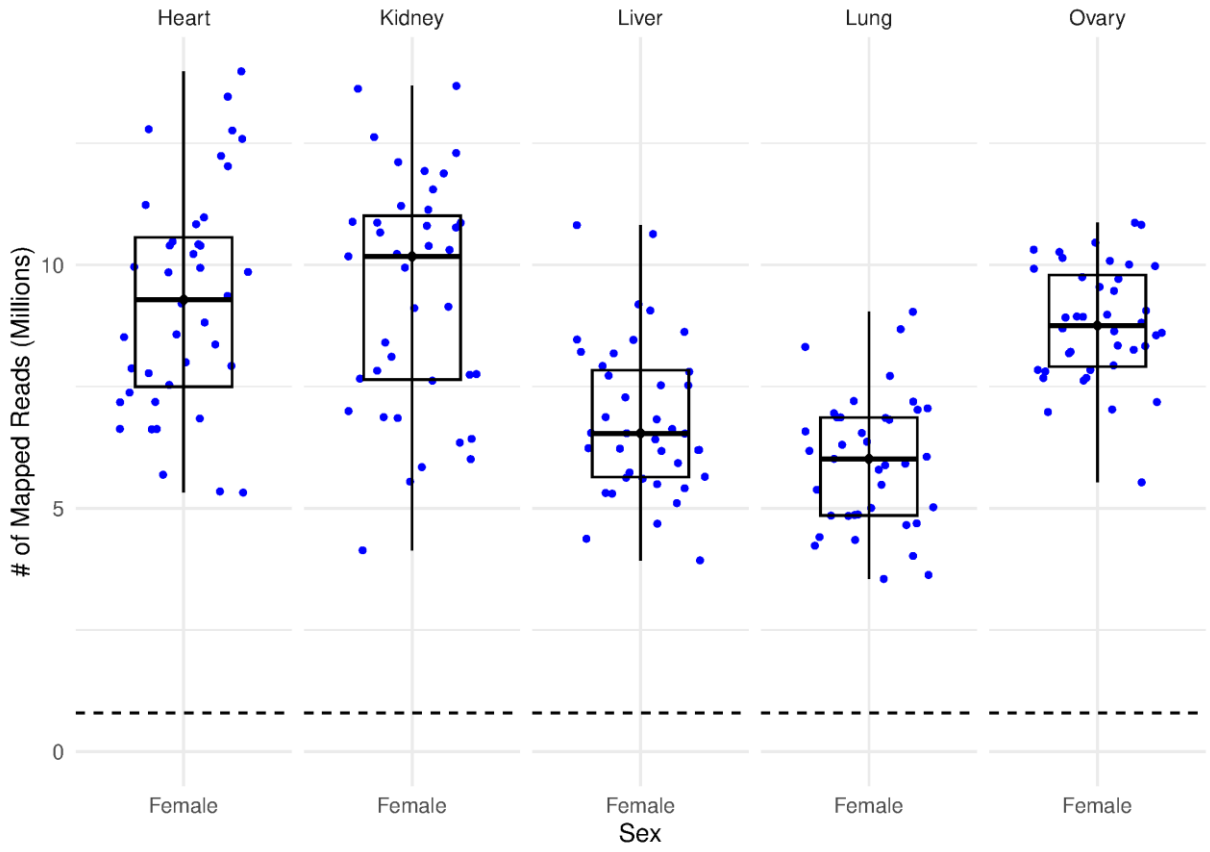
The quality statistics for the remaining sequencing samples are provided in Table_Apx A-7 and Table_Apx A-8 and Figure_Apx A-1 through Figure_Apx A-12.

Table_Apx A-7. Minimum and Median Sequencing Depth, Mapping Rate, and Probe Coverage Statistics by Tissue and Chemical in Rats

Sample Group		Sequencing Depth		Mapping Rate		Probe Coverage	
Tissue	Chemical	Minimum	Median	Minimum	Median	Minimum	Median
Heart	*o*-Dichlorobenzene	5323797	9285142	0.94	0.95	1975	2090
Kidney	*o*-Dichlorobenzene	4137944	10175693	0.96	0.96	2159	2293
Liver	*o*-Dichlorobenzene	3931765	6538397	0.97	0.97	1910	2013
Lung	*o*-Dichlorobenzene	3550740	6017102	0.92	0.95	2198	2269
Ovary	*o*-Dichlorobenzene	5533237	8752718	0.84	0.96	2206	2286
Heart	*p*-Dichlorobenzene	2867735	8805476	0.93	0.95	1855	2061
Kidney	*p*-Dichlorobenzene	1289233	7358082	0.93	0.95	2008	2269
Liver	*p*-Dichlorobenzene	4330479	7399112	0.95	0.97	1929	2033
Lung	*p*-Dichlorobenzene	4705453	9148207	0.88	0.95	2215	2288
Ovary	*p*-Dichlorobenzene	6519201	14558729	0.90	0.95	2233	2340

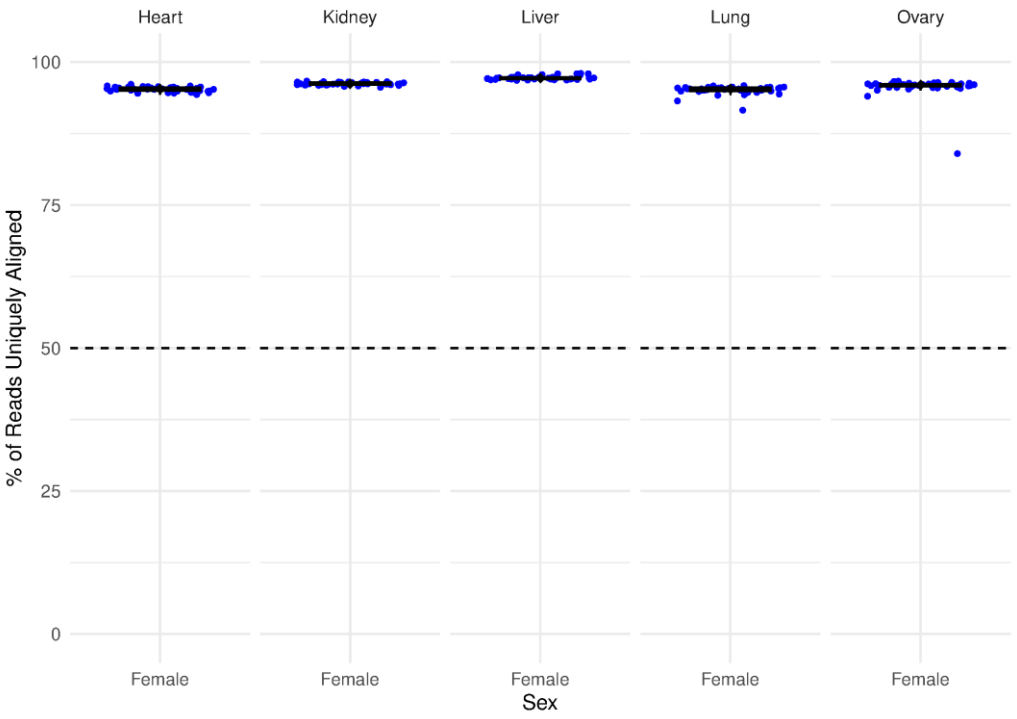
Table_Apx A-8. Minimum and Median Sequencing Depth, Mapping Rate, and Probe Coverage Statistics by Tissue and Chemical in Mice

Sample Group		Sequencing Depth		Mapping Rate		Probe Coverage	
Tissue	Chemical	Minimum	Median	Minimum	Median	Minimum	Median
Heart	*o*-Dichlorobenzene	6097609	8105181	0.95	0.96	2228	2323
Kidney	*o*-Dichlorobenzene	5788934	8564693	0.63	0.94	2508	2573
Liver	*o*-Dichlorobenzene	7359111	9259540	0.94	0.95	2358	2417
Lung	*o*-Dichlorobenzene	8912140	10401173	0.94	0.95	2530	2678
Ovary	*o*-Dichlorobenzene	3507206	4420311	0.90	0.95	2550	2623
Heart	*p*-Dichlorobenzene	8896369	12105516	0.95	0.96	2324	2417
Kidney	*p*-Dichlorobenzene	7336687	11269793	0.80	0.95	2561	2651
Liver	*p*-Dichlorobenzene	7880303	17036668	0.93	0.95	2374	2513
Lung	*p*-Dichlorobenzene	8056283	15678155	0.89	0.95	2626	2725
Ovary	*p*-Dichlorobenzene	3591754	7834463	0.83	0.95	2585	2728



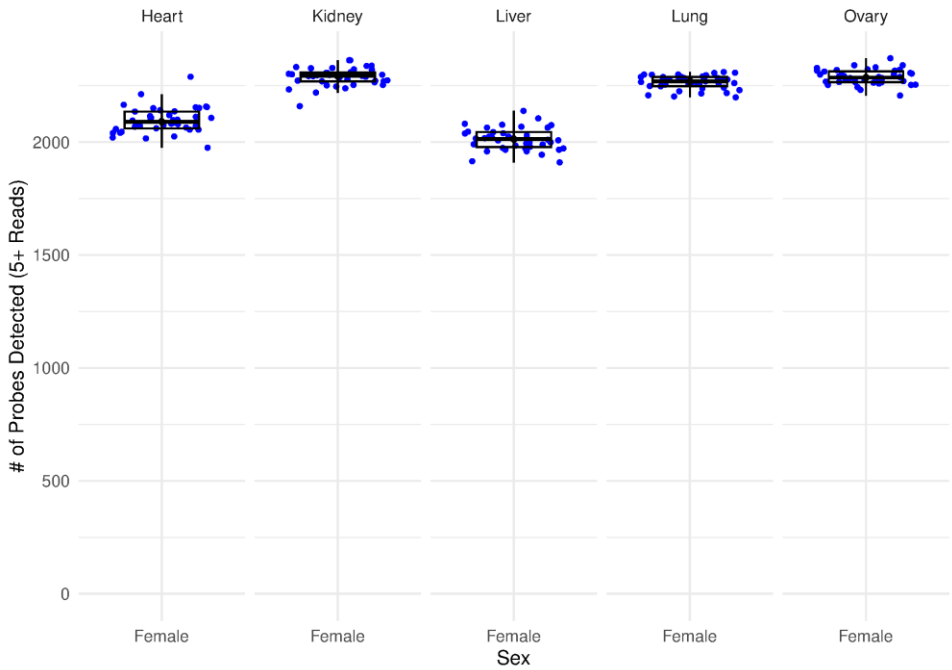
Figure_Apx A-1. Distribution of Sequencing Depth (Number of Uniquely Mapped Reads) for Each Sample in the Rat *o*-Dichlorobenzene Study, Grouped by Tissue

The horizontal dashed line represents the quality cutoff of 10% of the approximate median read depth.

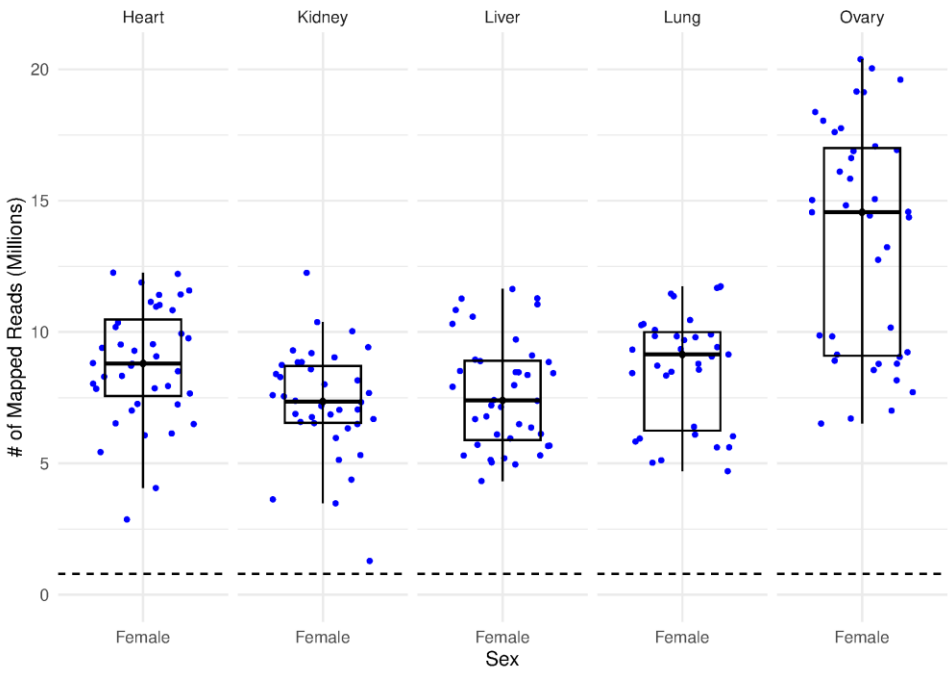


Figure_Apx A-2. Distribution of Mapping Rate (Percent of Reads Uniquely Aligned to Probes) for Each Sample in the Rat *o*-Dichlorobenzene Study, Grouped by Tissue

The horizontal dashed line represents the quality cutoff of 50% of uniquely aligned reads.

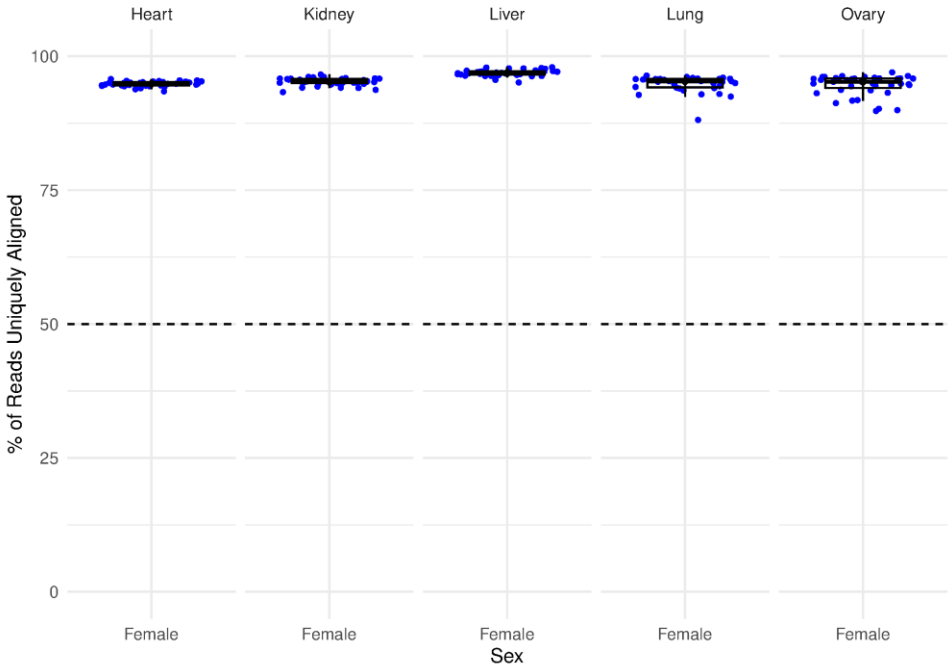


Figure_Apx A-3. Distribution of Probe Coverage (Number of Probes Detected with at Least 5 Reads) per Sample in the Rat *o*-Dichlorobenzene Study, Grouped by Tissue



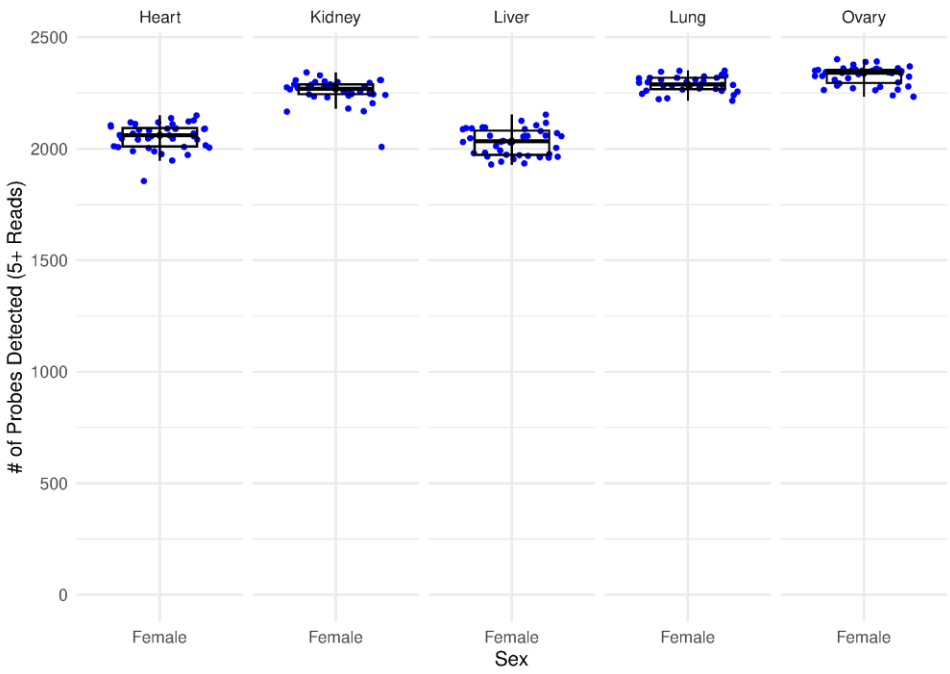
Figure_Apx A-4. Distribution of Sequencing Depth (Number of Uniquely Mapped Reads) for Each Sample in the Rat *p*-Dichlorobenzene Study, Grouped by Tissue

The horizontal dashed line represents the quality cutoff of 10% of the approximate median read depth.

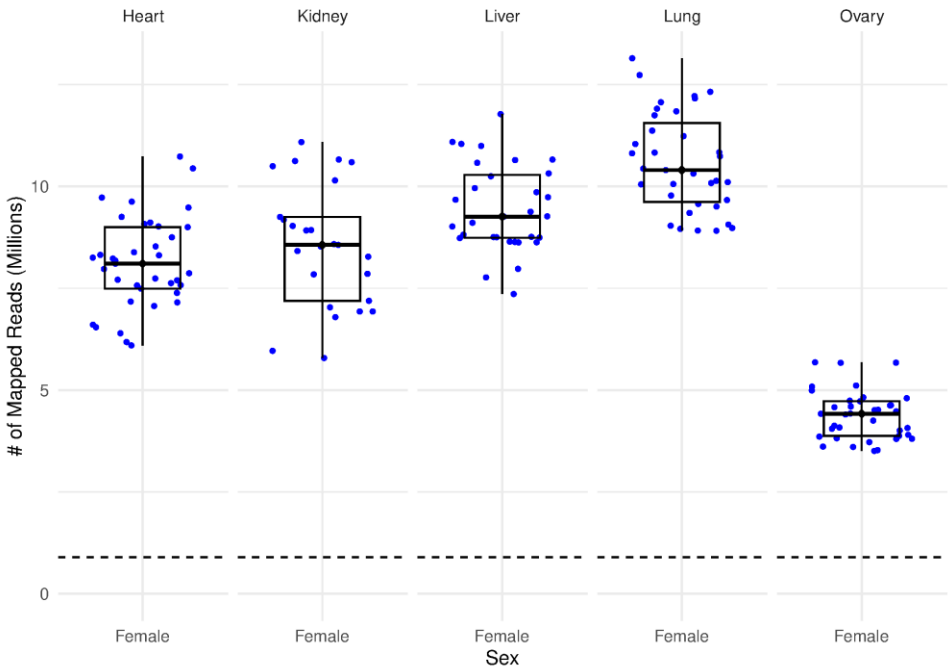


Figure_Apx A-5. Distribution of Sequencing Depth (Number of Uniquely Mapped Reads) for Each Sample in the Rat *p*-Dichlorobenzene Study, Grouped by Tissue

The horizontal dashed line represents the quality cutoff of 50% of uniquely aligned reads.

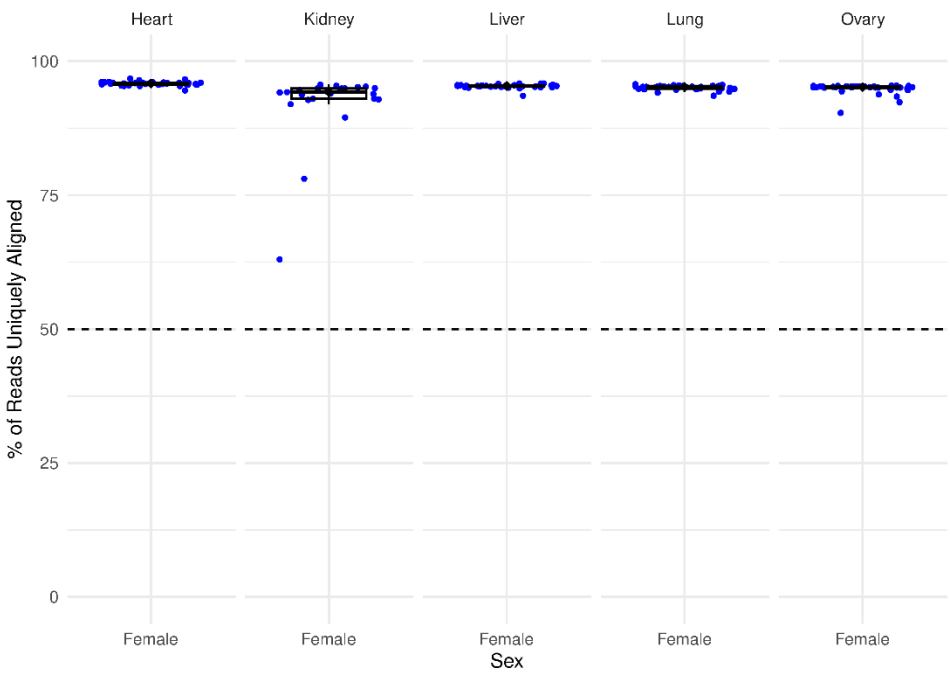


Figure_Apx A-6. Distribution of Sequencing Depth (Number of Uniquely Mapped Reads) for Each Sample in the Rat *p*-Dichlorobenzene Study, Grouped by Tissue



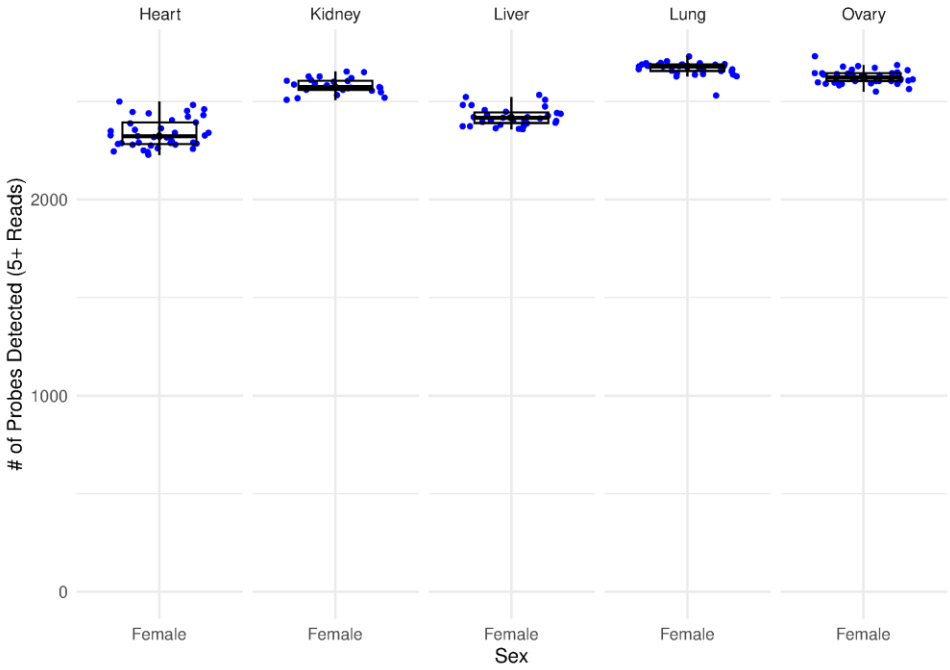
Figure_Apx A-7. Distribution of Sequencing Depth (Number of Uniquely Mapped Reads) for Each Sample in the Rat *p*-Dichlorobenzene Study, Grouped by Tissue

The horizontal dashed line represents the quality cutoff of 10% of the approximate median read depth.

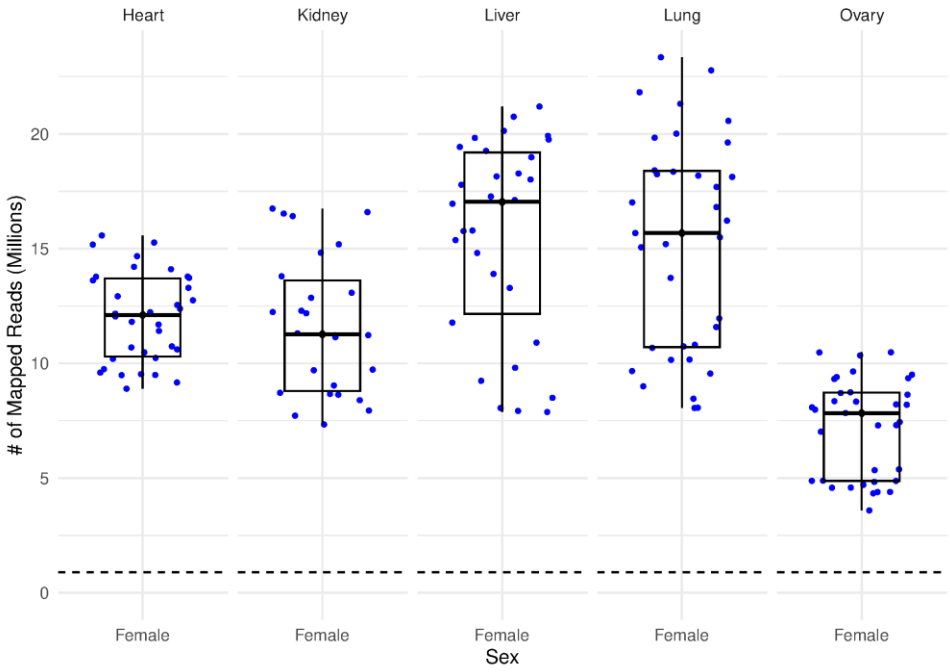


Figure_Apx A-8. Distribution of Mapping Rate (Percent of Reads Uniquely Aligned to Probes) for Each Sample in the Mouse *o*-Dichlorobenzene Study, Grouped by Tissue

The horizontal dashed line represents the quality cutoff of 50% of uniquely aligned reads.

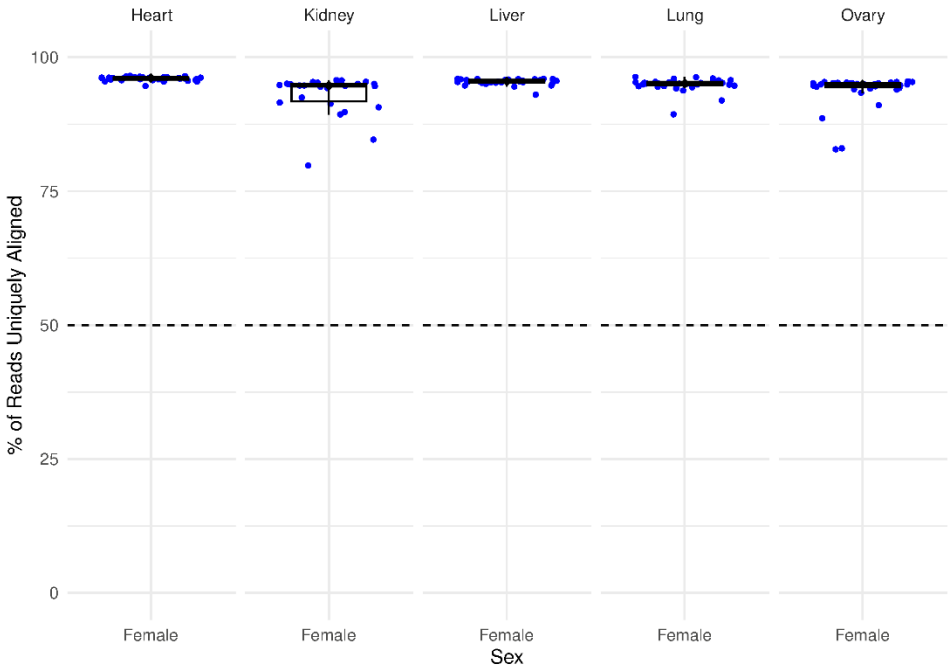


Figure_Apx A-9. Distribution of Probe Coverage (Number of Probes Detected with at Least 5 Reads) per Sample in the Mouse *o*-Dichlorobenzene Study, Grouped by Tissue



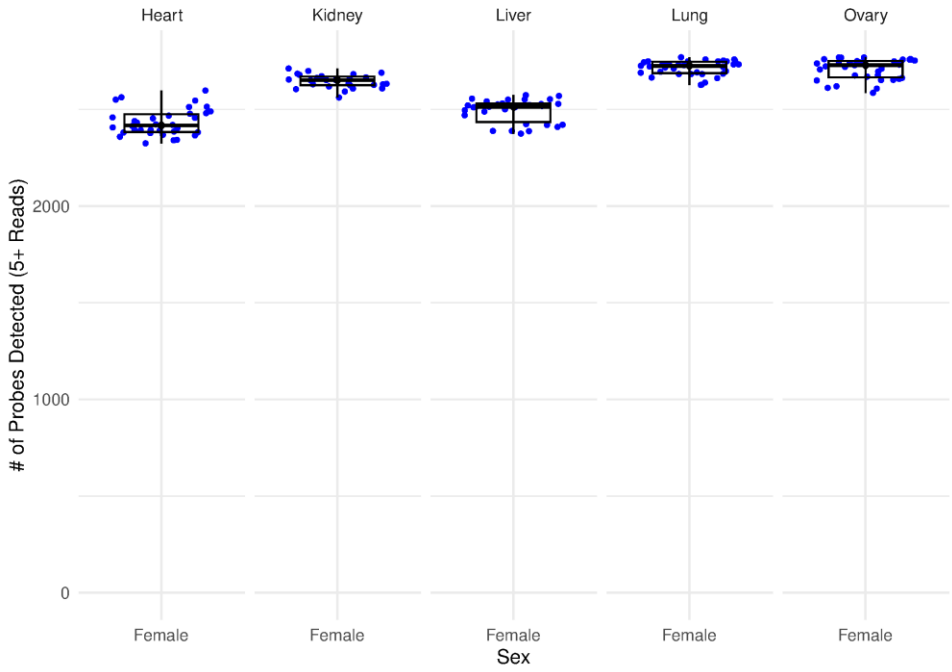
Figure_Apx A-10. Distribution of Sequencing Depth (Number of Uniquely Mapped Reads) for Each Sample in the Mouse *p*-Dichlorobenzene Study, Grouped by Tissue

The horizontal dashed line represents the quality cutoff of 10% of the approximate median read depth.



Figure_Apx A-11. Distribution of Mapping Rate (Percent of Reads Uniquely Aligned to Probes) for Each Sample in the Mouse *p*-Dichlorobenzene Study, Grouped by Tissue

The horizontal dashed line represents the quality cutoff of 50% of uniquely aligned reads.



Figure_Apx A-12. Distribution of Probe Coverage (Number of Probes Detected with at Least 5 Reads) per Sample in the Mouse *p*-Dichlorobenzene Study, Grouped by Tissue

A.3 Transcriptional Dose-Response Modeling with BMDExpress

Transcriptomic dose-response analyses were performed by EPA as described in the *Standard Methods for Development of EPA Transcriptomic Assessment Products* (U.S. EPA, 2024b). In brief, the dose-response modeling was performed independently on each probe, tissue, and chemical for both rats and mice using the software BMDExpress v2.3.0515 (released August 2024) and the nominal dose concentrations tested in the study. Count data are normalized to $\log_2$(CPM) with added pseudo-count of one as input into BMDExpress. For each dataset, an analysis of variance (ANOVA) pre-modeling test is used to confirm the number of probes that have a significant response with a false-discovery rate (FDR) less than 0.05 and suitable probes are pre-filtered prior to dose-response modeling with a William's Trent test ($p < 0.05$) and a mean absolute fold-change of 1.5× greater than vehicle controls in at least one dose group. Model fitting and BMD determination are performed for each probe passing the pre-filtering criteria:

- Linear, second degree polynomial, power (restricted to >= 1), Hill, second degree exponential, third degree exponential, fourth degree exponential, and fifth degree exponential models are used assuming constant variance.
- The model with the lowest Akaike information criteria is selected as the best fit model except in cases where the Hill model is selected but has a "k" parameter less than one-third the lowest dose. In this case, the Hill model is excluded from selection.
- The Benchmark Response (BMR) is set to 1.349.
- Probes meeting the following criteria are included for gene set analysis (see Appendix A.6 below):
    - BMD < highest tested dose
    - Model fit p-value > 0.1
    - BMD/BMDL < 20

A.4 Differential Gene Expression Analysis

Differential gene expression analysis of probe-level count data were performed by EPA using DESeq2 v1.44.0 (Love et al., 2014a) following a standardized HTTr pipeline that has been previously described (Bundy et al., 2024; Harrill et al., 2024; Harrill et al., 2021). Briefly, for each species, tissue, and chemical, samples for replicates of all doses were modeled jointly with matched vehicle control samples. Within each subset of samples used for modeling, probes with a mean read count < 5 were excluded. Size factors and dispersion were estimated using package defaults, and moderated $\log_2$ fold changes for each probe were estimated with "normal" shrinkage applied independently to each model contrast (*i.e.,* dose group vs control group). It should be noted that the number of samples in the mouse liver *o*-dichlorobenzene 1 ppm dose group (Table_Apx A-6) was below the minimum sample size recommended for DESeq2 analysis (n = 3), and other tissue dose groups are below the ideal sample size recommendation (n = 6) (Li et al., 2022).

A.5 Gene Set Dose-Response Modeling Analysis

Gene expression values were used by EPA for pathway/gene set analyses within each concentration using the eXploring Genomic Relations (XGR) R package (Fang et al., 2016) to analyze overexpression of Hallmark and Reactome subcollections within the Molecular Signatures Database (MSigDB). XGR enrichment analyses were performed using the human database with mouse and rat genes mapped to the human ortholog using an NCBI orthology mapping package, Orthology.eg.db (DOI: 10.18129/B9.bioc.Orthology.eg.db) and genome-wide annotations for mouse, rat, and human (DOI: 10.18129/B9.bioc.org.Mm.eg.db). Only genes with an FDR adjusted p-value less than or equal to 0.05 and absolute $\log_2$ fold change greater than or equal to $\log_2(1.5)$ were identified as differentially expressed and gene set background was constrained to probes detected and meeting the criteria for inclusion in differential gene expression analysis. Heatmaps were generated using the gplots R package (Warnes et al., 2024) showing the DESeq2 output $\log_2$ fold change for each gene annotated to highlighted pathways/gene sets and supersets in MSigDB.

Benchmark dose-response modeling using BMDExpress was performed by EPA and followed the same methods as described in the *Standard Methods for Development of EPA Transcriptomic Assessment Products* (U.S. EPA, 2024b). The resulting BMD gene results were exported and merged with species-specific MSigDB annotation files generated with the msigdbr R package (Dolgalev, 2025) using entrez gene IDs specific to each species (*i.e.,* rat and mouse). The MSigDB annotations were selected over GO terms for several reasons, including that MSigDB contains a curated enrichment set of GO terms that reduces redundancy, the genes comprised in the TempO-Seq S1500+ panel covered the MSigDB biological pathways, and it has previously been demonstrated that extrapolation from the S1500+ panels showed concordance with greater than 90% of the biological pathway/gene set space represented in MSigDB (Bushel et al., 2018; Mav et al., 2018). Summary BMDs were calculated for each MSigDB gene set where at least three genes were annotated and had a BMD, with the median BMDL of all genes within a gene set presented.

MSigDB gene sets were further categorized into superset gene sets representing apoptosis, cytotoxicity, inflammation, oxidative stress, and necrosis using the following string-matching terms: "apoptosis," "cytotox," "inflam," "oxidative," or "necrosis." Aggregated MSigDB gene sets consisting of a combination of any of the five superset terms were also created. For *p*-dichlorobenzene, an additional set of MSigDB superset gene sets was analyzed representing cell cycle, cell transport, inflammation, oxidative stress, and lipid metabolism using the following string-matching terms: "cell cycle," "cell trans," "inflam," "oxidative," and "lipid met." Aggregated MSigDB gene sets consisting of a combination of any of these five superset terms was also created. There are different numbers of genes

annotated in the rat and mouse MSigDB libraries utilized (12,432 for rats, 7,258 for mice), and different numbers of probes were included in the manifests from BioSpyder for the S1500+ array (2,654 probes for rats; 3,154 for mice). Furthermore, different numbers of probes and genes will meet inclusion criteria for analysis (*e.g.,* mean count >5). For these reasons, direct comparisons of total numbers of genes cannot be made between species, but rather the magnitude, direction, benchmark doses, and types of effects between species can be compared.

Two methods for determining the tPOD were calculated within each species and tissue: (1) the single gene set with the lowest (*i.e.,* minimum) BMDL median within each defined databases of gene sets was used to define the Lowest BMDL Median and (2) the 5th percentile of the distribution of gene sets or supersets within each defined database of gene sets was calculated to determine a 5th Percentile BMDL Median of the distribution. Both methods were applied to the GOBP[5], MSigDB, and MSigDB mechanism-informed supersets.

A.6 Species Comparison of CAR Biomarker Gene Set

For each of the identified CAR biomarker genes, the difference in log2 fold change of gene expression in liver tissue following o-dichlorobenzene or p-dichlorobenzene exposure between species relative to mouse was calculated by EPA according to Equation_Apx A-1. The equivalent dose groups for o-dichlorobenzene were 1 ppm, 10 ppm, 30 ppm, 100 ppm, and 250 ppm; for p-dichlorobenzene the equivalent dose groups were 1 ppm, 10 ppm, 50 ppm, 150 ppm, and 400 ppm.

$$log2(FC_M) - log2(FC_R)$$

Equation_Apx A-1. Log_2 Fold Change Difference in Gene Expression Between Species Relative to Mouse, Where *M* Is the Expression of the Gene in Mouse and *R* is the Expression of the Gene in Rat

[5] The Gene Ontology (https://geneontology.org/; accessed March 27, 2026) (GO) is a consortium of annotation data, ontologies, and tools to build gene ontology, assign ontology to gene/gene products, and develop software and databases to support those goals. GO's framework categorizes biological knowledge into three domains, with Biological Process describing the events within a biological activity (*e.g.,* immune response), Molecular Function describing specific biochemical activities of a gene product (*e.g.,* kinase activity), and Cellular Component describing the location where the gene product functions (*e.g.,* nucleus).

Appendix B EPA TRANSCRIPTOMIC ASSESSMENT PRODUCT (ETAP) ANALYSIS

B.1 Overview

The EPA developed the EPA Transcriptomic Assessment Product (ETAP) as a standard method to provide potency information for chemicals lacking traditional toxicity data (U.S. EPA, 2024a, b). The ETAP defines a prescriptive set of methods for conducting 5-day *in vivo* transcriptomic studies, with the goal to derive a transcriptomic reference value (TRV), defined as the estimate of a daily oral dose to the human population that is likely to have no appreciable risk of adverse non-cancer health effects over a lifetime, and was peer-reviewed by an independent Board of Scientific Counselors and implemented as an alternative testing approach in 2024 (BOSC, 2023). The TRV is experimentally determined from conversion of the $GOBP_{low}$ tPOD to human equivalent dose with the addition of a composite uncertainty factor of 300. The methods to derive this value are described in the *Standard Methods for Development of ETAPs* (U.S. EPA, 2024b). In brief, the ETAP requires daily oral exposure to a chemical for 5 days in both male and female rats with a minimum of eight dose levels plus a vehicle control group. Animals are sacrificed 24 hours after the final exposure and multiple tissues are collected for transcriptomic analysis using the targeted TempO-Seq s1500+ rat assay. Gene expression data are analyzed using the peer-reviewed, BMD modeling software BMDExpress version 2.3 (Phillips et al., 2019; Yang et al., 2007) to derive values across all collected tissues and sexes where further summarization is performed to identify the final value that is used to calculate the TRV. Because the chemicals for which ETAP was designed to be employed have no available toxicity data, the TRV uses a prescribed set of uncertainty factors to account for variability and uncertainty which are consistent with traditional human health assessments. Thus far, the EPA has completed ETAPs for several chemicals including multiple data-poor per- and polyfluoroalkyl substances (PFAS) (Mutlu et al., 2025; Brennan et al., 2024).

It is important to note that the ETAP was intended to be applied to substances with no existing or publicly accessible repeated-dose toxicity studies or human evidence suitable for use as a POD. In addition, the coordinated transcriptional changes used to identify the POD do not necessarily discriminate between specific hazards, adverse or adaptive effects, nor are they intended to infer a mechanism or MOA. However, the methods outlined in the ETAP provide a general framework for conducting 5-day *in vivo* transcriptomic studies that can be applied in other use cases. This includes examining transcriptomic responses following an alternative exposure route where traditional toxicity data are absent (*e.g.,* inhalation), examining additional tissues and species that lack toxicity data, or simply providing transcriptomic support for chemicals with traditional toxicity studies that are not considered data-poor. In this draft TSD, transcriptomic analyses to investigate MOA required a more comprehensive gene set database for enrichment analysis, BMD modeling, and tPOD derivation. MSigDB includes expert curated, well-annotated gene sets linked to biological states and processes, chemical/genetic perturbations, canonical molecular signaling pathways (and so on), including GO terms, and was therefore selected for these analyses. Changes in this approach also supported exploration of alternative methods to set the tPOD that may be better associated with reported apical effects such as the 5th percentile BMDL gene set. The 5th percentile BMDL is established for other EPA risk assessment methods as an appropriate regulatory value.

B.2 5-Day *In Vivo* Transcriptomic Study Design Considerations

The 5-day *in vivo* transcriptomic study design and analysis workflow presented in this draft TSD followed the *Standard Methods for Development of ETAPs* (U.S. EPA, 2024b), with the exceptions noted below:

- Exposure route was whole-body inhalation (as opposed to oral gavage in ETAP). Inhalation was chosen as the route of exposure since in the rodent cancer bioassays, inhalation studies in mice resulted in a greater tumor incidence than that observed in oral gavage studies.
- SD rats and B6D2F1/Crl mice were tested. Mice were chosen because of the increased incidence of liver tumors in both the gavage and inhalation bioassays.
- Only female animals tested. Females were more sensitive than males for both rats and mice based on dose and target organs.
- Five organs evaluated: heart, kidney, liver, lung, ovary. Tissue selection was based on known target tissues from guideline toxicity studies.
- The choice of inhalation as the route of exposure and the desire to run all exposures concurrently impacted the study design due to the capacity of the inhalation chambers. Thus, the following changes to the ETAP study design were implemented:
- Five dose levels plus a vehicle control for mice, and 6 dose levels plus a vehicle control for rats (as opposed to 8 dose levels plus a vehicle control in ETAP)
    - Sample size (n = 10) vehicle control animals (as opposed to n=8 in ETAP)
    - Sample size (n = 8) treatment group animals in the *o*-dichlorobenzene mouse 250 ppm dose group, and (n = 5) treatment group animals in all other *o*-dichlorobenzene and *p*-dichlorobenzene dose groups for rats and mice (as opposed to n = 5 in ETAP for all dose groups)
- Animal age of 6 to 10 weeks post acclimation at time of exposure (as opposed to 8–10 weeks post acclimation in ETAP)
- Preheated nitrogen gas was used as the dosing vehicle as opposed to propylene glycol in the standard methods based on the choice of inhalation as the exposure route.
- Sequencing read depth cutoff threshold of 800,000 mapped reads for rats and 900,000 mapped reads for mice was used (as opposed to 100,000 mapped reads, *i.e.,* 10% of the target read depth, in ETAP)
    - The target sequencing depth requested by NIEHS DTT was 1.5 million reads**,** however the sequencing instrument used returned more reads than anticipated (≈8–9 million median reads). Due to this discrepancy, the approximate median sequencing depth for each species was used as a proxy for the target read depth rather than the requested target read depth.

Detailed methods for the study performed by NIEHS DTT for both *o*-dichlorobenzene and *p*-dichlorobenzene are publicly available (NIEHS, 2025a, b).

B.3 Transcriptomic Data Analysis and GO Biological Process Summarization

B.3.1 Transcriptional Changes

Pre-modeling dataset evaluation was performed to determine where there was adequate signal. Note that multiple kidney samples from mice had evidence of tissue contamination and were excluded from analysis (see Table_Apx A-2 in Appendix A.2); however, the remaining number of samples across the vehicle controls and dose groups for both *o*-dichlorobenzene and *p*-dichlorobenzene were above the minimum thresholds required to proceed with dose-response modeling and subsequent analysis (U.S. EPA, 2024b). All tissues passed the ANOVA cut-off of at least one gene with false discovery rate (FDR) corrected p-value less than 0.05 for BMD modeling. The number of dose-responsive genes from female rats and mice, respectively, that passed the William's Trend testing pre-modeling filter varied

across species and tissues for *o*-dichlorobenzene (Table_Apx B-1 and Table_Apx B-2) and *p*-dichlorobenzene (Table_Apx B-3 and Table_Apx B-4).

Table_Apx B-1. Number of Dose-Responsive Genes Following Treatment with *o*-Dichlorobenzene in Female Rats[a]

Tissue	Species	Female
Heart	Rat	246
Kidney	Rat	302
Liver	Rat	1123
Lung	Rat	188
Ovary	Rat	176
[a] Based on Williams Trend test p-value < 0.05 and |Fold change| > 1.5.		

Table_Apx B-2. Number of Dose-Responsive Genes Following Treatment with *o*-Dichlorobenzene in Female Mice[a]

Tissue	Species	Female
Heart	Mouse	109
Kidney	Mouse	214
Liver	Mouse	784
Lung	Mouse	341
Ovary	Mouse	211
[a] Based on Williams Trend test p-value < 0.05 and |Fold change| > 1.5.		

Table_Apx B-3. Number of Dose-Responsive Genes Following Treatment with *p*-Dichlorobenzene in Female Rats[a]

Tissue	Species	Female
Heart	Rat	106
Kidney	Rat	136
Liver	Rat	351
Lung	Rat	201
Ovary	Rat	209
[a] Based on Williams Trend test p-value < 0.05 and |Fold change| > 1.5.		

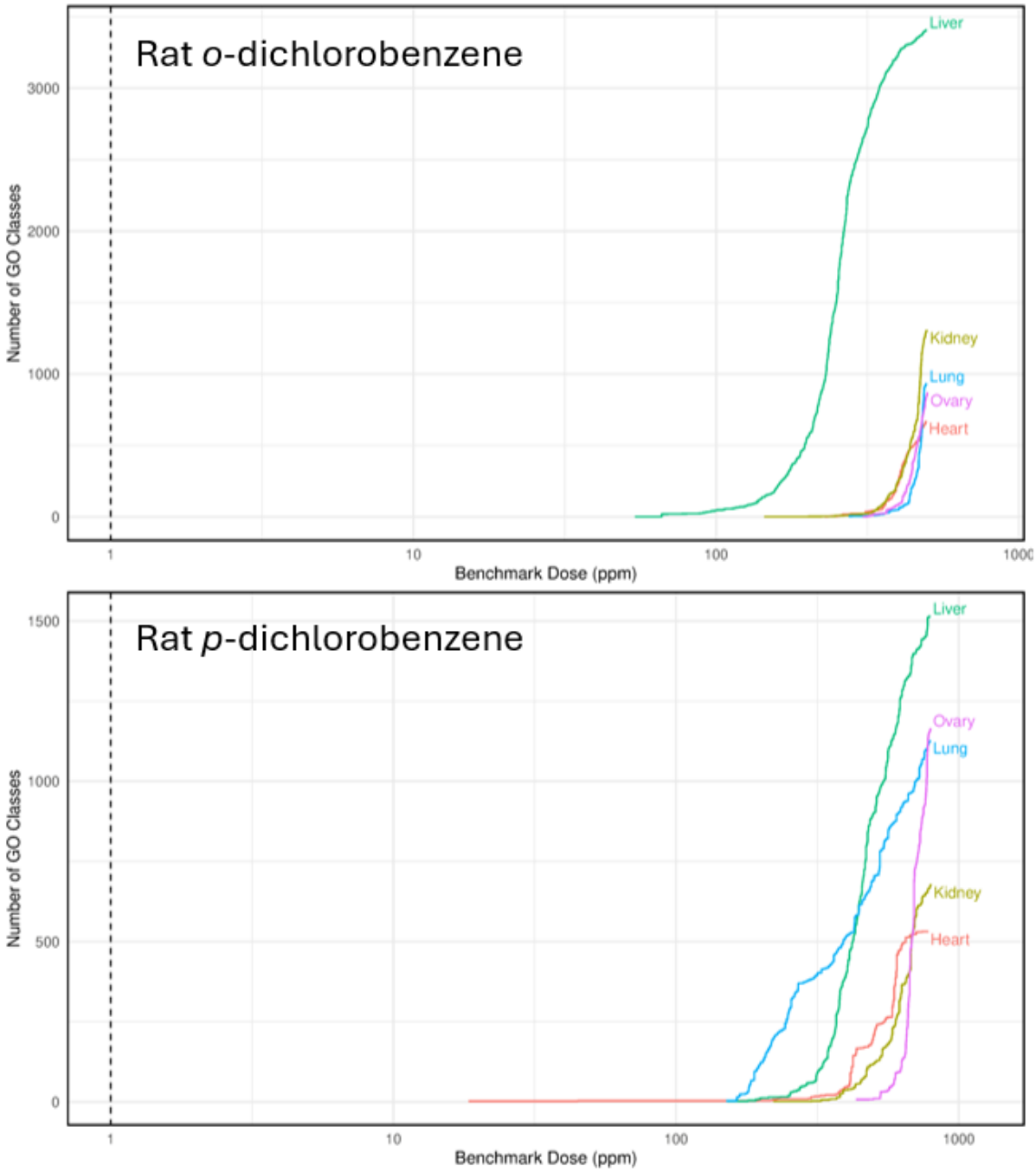
Table_Apx B-4. Number of Dose-Responsive Genes Following Treatment with *p*-Dichlorobenzene in Female Mice[a]

Tissue	Species	Female
Heart	Mouse	310
Kidney	Mouse	85
Liver	Mouse	511
Lung	Mouse	112
Ovary	Mouse	65

[a] Based on Williams Trend test p-value < 0.05 and |Fold change| > 1.5.

B.3.2 GOBP Dose-Response Modeling

Dose-response modeling and the $GOBP_{low}$ tPOD was calculated as described in Appendix A.3 and A.5, respectively, which followed the *Standard Methods for Development of ETAPs* (U.S. EPA, 2024b). For the *o*-dichlorobenzene study in female rats, the liver had the lowest Gene Ontology Biological Process (GOBP) tPOD value across tissues (Figure_Apx B-1; Table_Apx B-5). The GO biological process class was positive regulation of cytokinesis (GO:0032467) with a median BMD value of 53.9 ppm and an associated median BMDL value of 41.1 ppm (Table_Apx B-5).



Figure_Apx B-1. Accumulation Plots of GO Biological Process Classes by Median Benchmark Dose Value for Each Tissue in Female Rats Exposed to *o*-Dichlorobenzene (Top) and *p*-Dichlorobenzene (Bottom)

Table_Apx B-5. Lowest GO Biological Process Class Median Benchmark Dose Values Across Tissues in Female Rats Treated with *o*-Dichlorobenzene

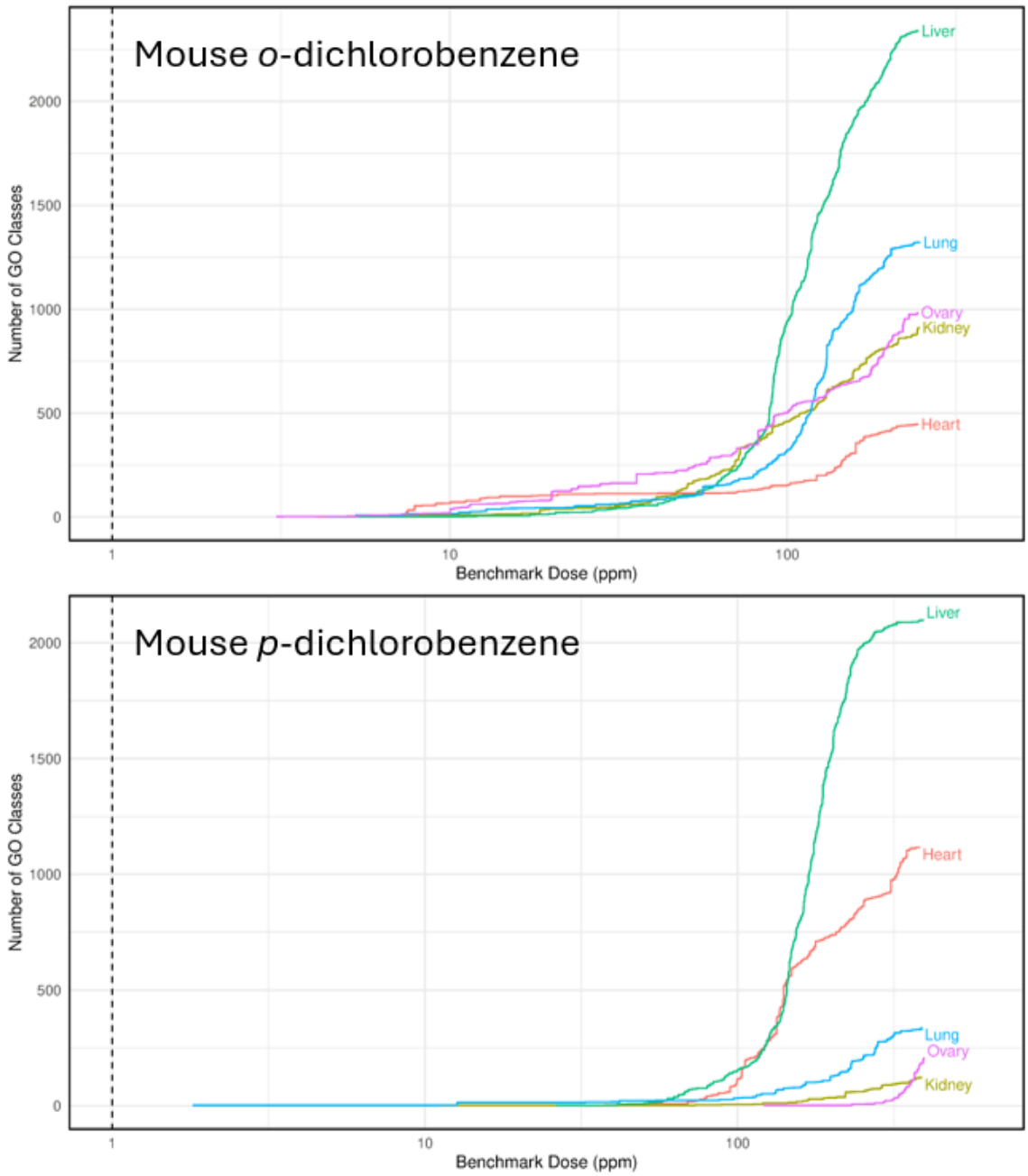
Tissue	GO Accession	GO Biological Process Class	# of Genes with BMD	Median BMD (ppm)	Median BMDL (ppm)
Heart	GO:0061515	myeloid cell development	3	229	107
Kidney	GO:0016052	carbohydrate catabolic process	3	144	116
Liver	GO:0032467	positive regulation of cytokinesis	3	53.9	41.1
Lung	GO:0006090	pyruvate metabolic process	3	274	205
Ovary	GO:0043271	negative regulation of monoatomic ion transport	3	305	224

For the *p*-dichlorobenzene study in female rats, the heart had the $GOBP_{low}$ tPOD value across tissues (Figure_Apx B-1; Table_Apx B-6). The GO biological process class was positive regulation of ERK1 and ERK2 cascade (GO:0070374) with a median BMD value of 18.5 ppm and an associated median BMDL value of 1.80 ppm (Table_Apx B-6).

Table_Apx B-6. Lowest GO Biological Process Class Median Benchmark Dose Values Across Tissues in Female Rats Treated with *p*-Dichlorobenzene

Tissue	GO Accession	GO Biological Process Class	# of Genes with BMD	Median BMD (ppm)	Median BMDL (ppm)
Heart	GO:0070374	positive regulation of ERK1 and ERK2 cascade	3	18.5	1.80
Kidney	GO:0030522	intracellular receptor signaling pathway	3	221	179
Liver	GO:0098656	monoatomic anion transmembrane transport	3	152	99.3
Lung	GO:0006486	protein glycosylation	3	151	101
Ovary	GO:0045773	positive regulation of axon extension	3	433	317

For the *o*-dichlorobenzene study in female mice, the ovary had the $GOBP_{low}$ tPOD value across tissues (Figure_Apx B-2; Table_Apx B-7). The GO biological process class was positive regulation of muscle contraction (GO:0045933) with a median BMD value of 3.07 ppm and an associated median BMDL value of 0.73 ppm (Table_Apx B-7).



Figure_Apx B-2. Accumulation Plots of GO Biological Process Classes by Median Benchmark Dose Value for Each Tissue in Female Mice Exposed to *o*-Dichlorobenzene (Top) and *p*-Dichlorobenzene (Bottom)

Table_Apx B-7. Lowest GO Biological Process Class Median Benchmark Dose Values Across Tissues in Female Mice Treated with *o*-Dichlorobenzene

Tissue	GO Accession	GO Biological Process Class	# of Genes with BMD	Median BMD (ppm)	Median BMDL (ppm)
Heart	GO:0006730	one-carbon metabolic process	3	4.03	1.09
Kidney	GO:0007623	circadian rhythm	3	4.15	1.01
Liver	GO:0071397	cellular response to cholesterol	3	4.65	1.22
Lung	GO:0001843	neural tube closure	3	4.35	1.21
Ovary	GO:0045933	positive regulation of muscle contraction	3	3.07	0.73

For the *p*-dichlorobenzene study in female mice, the lung had the $GOBP_{low}$ tPOD value across tissues (Figure_Apx B-2, Table_Apx B-8). The GO biological process class was cellular response to organic cyclic compound (GO:0071407) with a median BMD value of 1.81 ppm and an associated median BMDL value of 0.43 ppm (Table_Apx B-8).

Table_Apx B-8. Lowest GO Biological Process Class Median Benchmark Dose Values Across Tissues in Female Mice Treated with *p*-Dichlorobenzene

Tissue	GO Accession	GO Biological Process Class	# of Genes with BMD	Median BMD (ppm)	Median BMDL (ppm)
Heart	GO:0030183	B cell differentiation	3	6.61	1.03
Kidney	GO:0006810	transport	3	9.36	3.71
Liver	GO:0048714	positive regulation of oligodendrocyte differentiation	3	26.2	7.73
Lung	GO:0071407	cellular response to organic cyclic compound	3	1.81	0.43
Ovary	GO:0015031	protein transport	3	121	61.5

B.3.3 Final $GOBP_{low}$ tPOD

The $GOBP_{low}$ tPOD values for the *o*-dichlorobenzene and *p*-dichlorobenzene studies in female rats and mice can be found in Table_Apx B-9.

Table_Apx B-9. Transcriptomic Point of Departure Values for *o*-Dichlorobenzene and *p*-Dichlorobenzene Studies in Female Rats and Mice

Chemical	Species	Point of Departure
o-Dichlorobenzene	Rat	41.1 ppm
o-Dichlorobenzene	Mouse	0.73 ppm
p-Dichlorobenzene	Rat	1.80 ppm
p-Dichlorobenzene	Mouse	0.43 ppm

Appendix C *p*-DICHLOROBENZENE tPOD ANALYSIS

C.1 tPOD Determination Analysis for *p*-Dichlorobenzene Using *o*-Dichlorobenzene Superset Terms

The same analysis of MSigDB superset terms determined for *o*-dichlorobenzene (apoptosis, cytotoxicity, inflammation, oxidative stress, necrosis) was applied to *p*-dichlorobenzene. The *o*-dichlorobenzene analysis demonstrated that the MSigDB combined superset was the most useful for the *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene* (U.S. EPA, 2026a). To increase readability of the report, only the combined MSigDB superset tPODs, the $MSigDB_{low}$ and $MSigDB_{0.05}$ are presented for *p*-dichlorobenzene rather than showing each superset-specific BMDL value. Note that the $GOBP_{low}$ and $GOBP_{0.05}$ were also calculated.

Within the mouse liver, the $GOBP_{low}$ tPOD was 7.73 ppm and the $GOBP_{0.05}$ tPOD was 54.76. The MSigDB $SUPER_{low}$ was 30.25 ppm for the GOBP term "regulation of tumor necrosis factor mediated signaling." The $MSigDB_{0.05}$ tPOD within the combined MSigDB superset was 71.4 ppm. A full table of mouse liver BMD results for *p*-dichlorobenzene can be found in Table_Apx C-1, and accumulation plots for the combined MSigDB superset compared with the full landscape of gene sets in MSigDB are shown in Figure_Apx C-1 (MSigDB $SUPER_{low}$) and Figure_Apx C-2 (MSigDB $SUPER_{0.05}$).

Within the mouse lung, the $GOBP_{low}$ tPOD was 0.43 ppm and the $GOBP_{0.05}$ tPOD was 29.94. The MSigDB $SUPER_{low}$ was 122.54 ppm for the GOBP term "reactive oxygen species metabolic process." The $MSigDB_{0.05}$ tPOD within the combined MSigDB superset was 128.68 ppm. A full table of mouse lung BMD results for *p*-dichlorobenzene can be found in Table_Apx C-2, and accumulation plots for the combined MSigDB superset compared with the full landscape of gene sets in MSigDB are shown in Figure_Apx C-1 (MSigDB $SUPER_{low}$) and Figure_Apx C-2(MSigDB $SUPER_{0.05}$).

Within the mouse kidney, the $GOBP_{low}$ tPOD was 3.71 ppm and the $GOBP_{0.05}$ tPOD was 57.40. Within the combined set of aggregated supersets, the MSigDB $SUPER_{low}$ was 164.45 ppm for the GOBP term "inflammatory response." The $MSigDB_{0.05}$ tPOD within the combined MSigDB superset was 164.45 ppm. A full table of mouse kidney BMD results for *p*-dichlorobenzene can be found in Table_Apx C-3, and accumulation plots for the combined MSigDB superset compared with the full landscape of gene sets in MSigDB are shown in Figure_Apx C-1 (MSigDB $SUPER_{low}$) and Figure_Apx C-2 (MSigDB $SUPER_{0.05}$).

Table_Apx C-1. BMD Modeling Results of Targeted Gene Sets Relating to Apical Effects from *p*-Dichlorobenzene Exposure (Apoptosis, Cytotoxicity, Inflammation, Oxidative Stress, Necrosis) Within Mouse Liver

	Gene Set Accession	Lowest BMDL Median[a] (ppm)	5th Percentile BMDL Median[b] (ppm)
GOBP	GO:0048714	7.73	54.76
MSigDB[c]	GO:0048714	7.73	54.41
MSigDB Combined Superset[d]	GO:0010803	30.25 (apoptosis, necrosis)	71.4

[a] The lowest BMDL median for GOBP represents the BMDL of the lowest GO term rank ordered by BMD Median.
[b] The 5th percentile BMDL Median for GOBP represents the 5th percentile of GO terms rank ordered by BMDL Median.
[c] The full MSigDB collection of gene sets.
[d] The Combined MSigDB Superset represents a consensus of multiple individual gene sets associated with a specific biological response. For this evaluation, cytotoxicity, apoptosis, inflammation, oxidative stress, and necrosis were prioritized.
The MSigDB and MSigDB superset results presented here are rank ordered by gene set-level BMDL Median and report the lowest and distribution-based 5th percentile gene set BMDL Medians for comparison.
For gene set accessions, the "GO" prefix refers to "Gene Ontology" accession identifiers, while the "MM" prefix refers to "mouse MSigDB" accession identifiers.

Table_Apx C-2. BMD Modeling Results of Targeted Gene Sets Relating to Apical Effects from *p*-Dichlorobenzene Exposure (Apoptosis, Cytotoxicity, Inflammation, Oxidative Stress, Necrosis) Within Mouse Lung

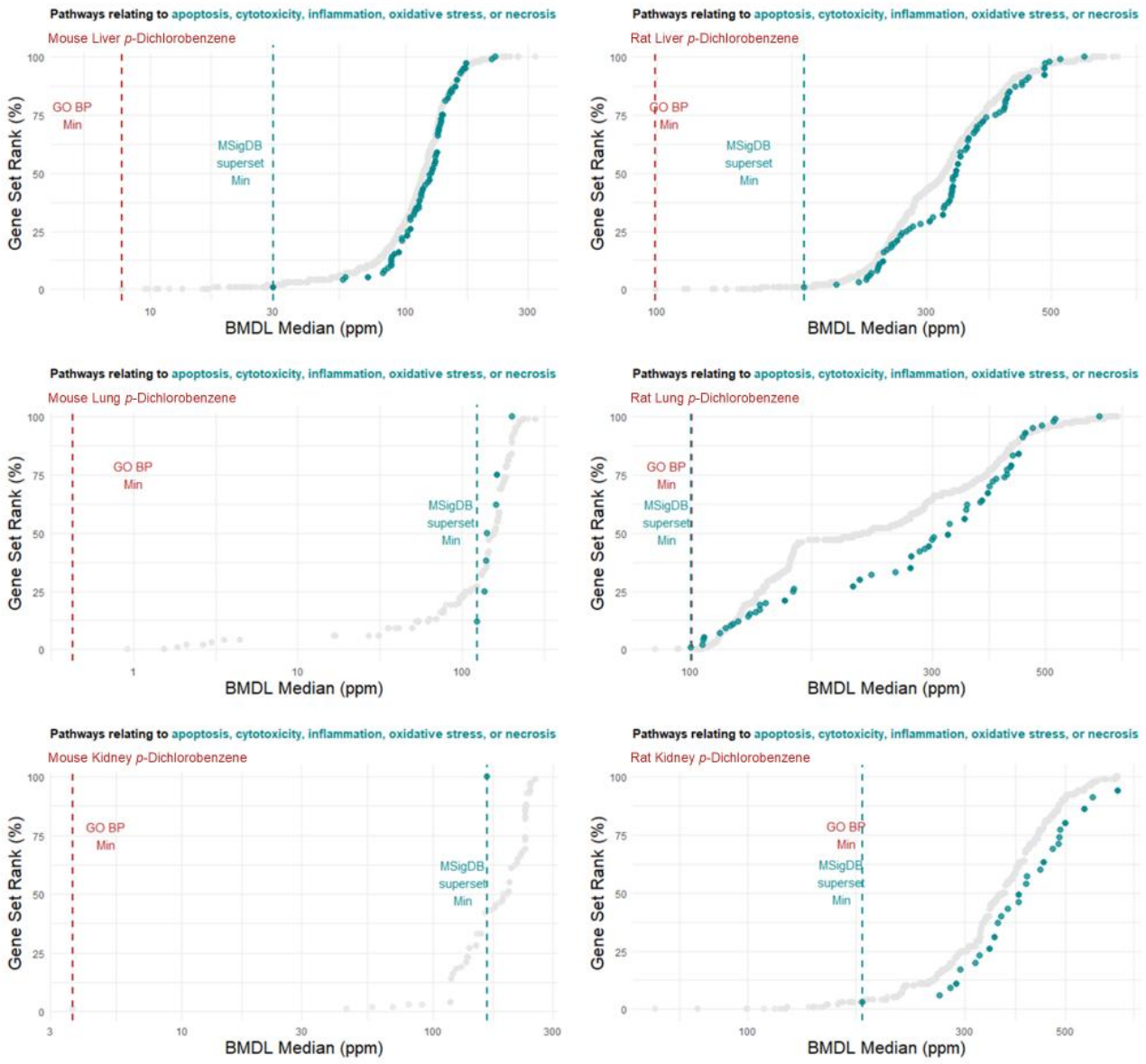
	Gene Set Accession	Lowest BMDL Median[a] (ppm)	5th Percentile BMDL Median[b] (ppm)
GOBP	GO:0071407	0.43	29.94
MSigDB[c]	GO:0046903	0.92	4.43
MSigDB Combined Superset[d]	MM9957	122.54 (oxidative stress)	128.68

[a]The lowest BMDL median for GOBP represents the BMDL of the lowest GO term rank ordered by BMD Median.
[b]The 5th percentile BMDL Median for GOBP represents the 5th percentile of GO terms rank ordered by BMDL Median.
[c]The full MSigDB collection of gene sets.
[d]The Combined MSigDB Superset represents a consensus of multiple individual gene sets associated with a specific biological response. For this evaluation, cytotoxicity, apoptosis, inflammation, oxidative stress, and necrosis were prioritized.
The MSigDB and MSigDB superset results presented here are rank ordered by gene set-level BMDL Median and report the lowest and distribution-based 5th percentile gene set BMDL Medians for comparison.
For gene set accessions, the "GO" prefix refers to "Gene Ontology" accession identifiers, while the "MM" prefix refers to "mouse MSigDB" accession identifiers.

Table_Apx C-3. BMD Modeling Results of Targeted Gene Sets Relating to Apical Effects from *p*-Dichlorobenzene Exposure (Apoptosis, Cytotoxicity, Inflammation, Oxidative Stress, Necrosis) Within Mouse Kidney

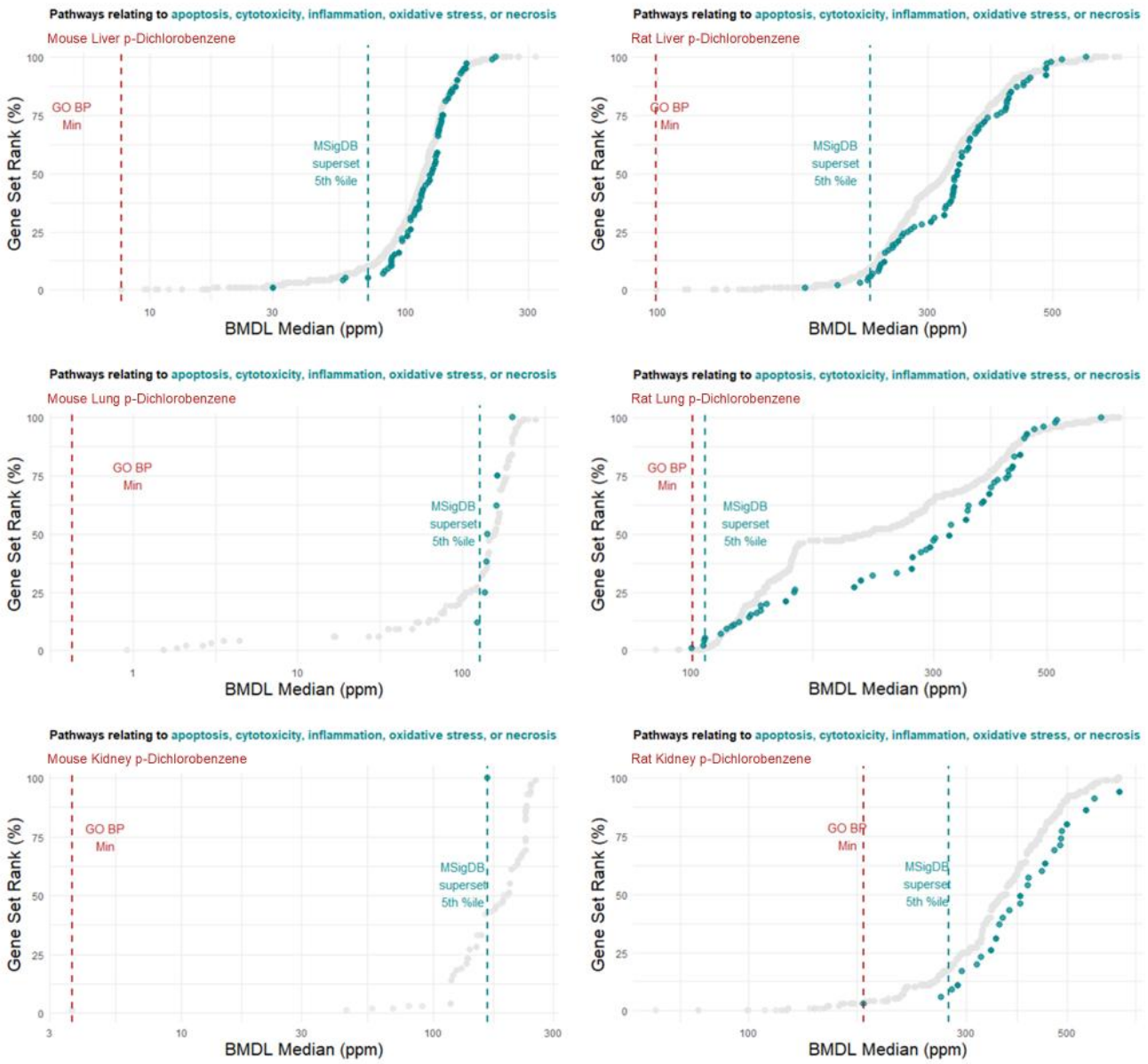
	Gene Set Accession	Lowest BMDL Median[a] (ppm)	5th Percentile BMDL Median[b] (ppm)
GOBP	GO:0006810	3.71	57.40
MSigDB[c]	GO:0015711	3.71	117.34
MSigDB Combined Superset	MM4923	164.45 (inflammation)	164.45

[a] The lowest BMDL median for GOBP represents the BMDL of the lowest GO term rank ordered by BMD Median.
[b] The 5th percentile BMDL Median for GOBP represents the 5th percentile of GO terms rank ordered by BMDL Median.
[c] The $MSigDB_{low}$ gene set, considering the full landscape of MSigDB gene sets.
[d] The Combined MSigDB Superset represents a consensus of multiple individual gene sets associated with a specific biological response. For this evaluation, cytotoxicity, apoptosis, inflammation, oxidative stress, and necrosis were prioritized.
The MSigDB and MSigDB superset results presented here are rank ordered by gene set-level BMDL Median and report the lowest and 5th percentile gene set BMDL Medians for comparison. For gene set accessions, the "GO" prefix refers to Gene Ontology accession identifiers, while the "MM" prefix refers to mouse MSigDB accession identifiers.



Figure_Apx C-1. Mouse and Rat Liver, Lung, and Kidney Accumulation Plots Showing the Gene Set Ranks (%)

All gene sets in the MSigDB database are shown with grayed circles; each circle represents a distinct MSigDB gene set term. The combined superset with any term from apoptosis, cytotoxicity, inflammation, necrosis, and oxidative stress, and including at least three genes with BMD meeting all inclusion criteria, is shown against the landscape of all gene sets in color, with each subsequent plot highlighting a specific subset of those gene sets. The vertical dashed red line highlights the $GOBP_{low}$ tPOD, and the other highlights the MSigDB $SUPER_{low}$ tPOD for the combined MSigDB superset covering the terms "apoptosis," "cytotox," "inflam," "oxidative," and "necrosis."



Figure_Apx C-2. Rat Kidney Accumulation Plots Showing the Gene Set Ranks (%)
All gene sets in the MSigDB database are shown with grayed circles; each circle represents a distinct MSigDB gene set term. The combined superset with any term from apoptosis, cytotoxicity, inflammation, necrosis, and oxidative stress, and including at least three genes with BMD meeting all inclusion criteria, is shown against the landscape of all gene sets in color, with each subsequent plot highlighting a specific subset of those gene sets. The vertical dashed red line highlights the $GOBP_{low}$ tPOD, and the other highlights the MSigDB $SUPER_{0.05}$ tPOD for the combined MSigDB superset covering the terms "apoptosis," "cytotox," "inflam," "oxidative," and "necrosis."

Within the rat liver, the $GOBP_{low}$ tPOD was 99.3 ppm and the $GOBP_{0.05}$ tPOD was 228.82. Within the combined set of aggregated supersets, the MSigDB $SUPER_{low}$ was 182.12 ppm for the GOBP term "ferroptosis." The $MSigDB_{0.05}$ tPOD within the combined MSigDB superset was 237.88 ppm. A full table of rat liver BMD results for *p*-dichlorobenzene can be found in Table_Apx C-4, and accumulation plots for the combined MSigDB superset compared with the full landscape of gene sets in MSigDB are shown in Figure_Apx C-1 (MSigDB $SUPER_{low}$) and Figure_Apx C-2 (MSigDB $SUPER_{0.05}$).

Within the rat lung, the $GOBP_{low}$ tPOD was 101 ppm and the $GOBP_{0.05}$ tPOD was 120.43. Within the combined set of aggregated supersets, the MSigDB $SUPER_{low}$ 100.37 ppm for the Graessmann curated term "apoptosis by serum deprivation." The $MSigDB_{0.05}$ tPOD within the combined MSigDB superset was 112.9 ppm. A full table of rat lung BMD results for *p*-dichlorobenzene can be found in Table_Apx C-5, and accumulation plots for the combined MSigDB superset compared with the full landscape of gene sets in MSigDB are shown in Figure_Apx C-1(MSigDB $SUPER_{low}$) and Figure_Apx C-2 (MSigDB $SUPER_{0.05}$).

Within the rat kidney, the $GOBP_{low}$ tPOD was 179 ppm and the $GOBP_{0.05}$ tPOD was 267.28. Within the combined set of aggregated supersets, the MSigDB $SUPER_{low}$ was 178.91 ppm for the Immunologic Signature curated term "ELF4 knockout vs wildtype activated CD8 T-cell." The $MSigDB_{0.05}$ tPOD within the combined MSigDB superset was 266.11 ppm. A full table of rat kidney BMD results for *p*-dichlorobenzene can be found in Table_Apx C-6, and accumulation plots for the combined MSigDB superset compared with the full landscape of gene sets in MSigDB are shown in Figure_Apx C-1(MSigDB $SUPER_{low}$) and Figure_Apx C-2 (MSigDB $SUPER_{0.05}$).

Table_Apx C-4. BMD Modeling Results of Targeted Gene Sets Relating to Apical Effects from *p*-Dichlorobenzene Exposure (Apoptosis, Cytotoxicity, Inflammation, Oxidative Stress, Necrosis) Within Rat Liver

	Gene Set Accession	Lowest BMDL Median[a] (ppm)	5th Percentile BMDL Median[b] (ppm)
GOBP	GO:0098656	99.3	228.82
MSigDB[c]	GO:0006820	99.34	222.62
MSigDB Combined Superset[d]	GO:0097707	182.12 (apoptosis)	237.88

[a] The lowest BMDL median for GOBP represents the BMDL of the lowest GO term rank ordered by BMD Median.
[b] The 5th percentile BMDL Median for GOBP represents the 5th percentile of GO terms rank ordered by BMDL Median.
[c] The full MSigDB collection of gene sets.
[d] The Combined MSigDB Superset represents a consensus of multiple individual gene sets associated with a specific biological response. For this evaluation, cytotoxicity, apoptosis, inflammation, oxidative stress, and necrosis were prioritized.
The MSigDB and MSigDB superset results presented here are rank ordered by gene set-level BMDL Median and report the lowest and distribution-based 5th percentile gene set BMDL Medians for comparison.
For gene set accessions, the "GO" prefix refers to Gene Ontology accession identifiers, while the "M" prefix refers to MSigDB accession identifiers that were mapped via orthology from human gene sets since there are no native rat gene sets available in MSigDB.

Table_Apx C-5. BMD Modeling Results of Targeted Gene Sets Relating to Apical Effects from *p*-Dichlorobenzene Exposure (Apoptosis, Cytotoxicity, Inflammation, Oxidative Stress, Necrosis) Within Rat Lung

	Gene Set Accession	Lowest BMDL Median[a] (ppm)	5th Percentile BMDL Median[b] (ppm)
GOBP	GO:0006486	101	120.43
MSigDB[c]	M6958	85.56	113.96
MSigDB Combined Superset[d]	M1098	100.37 (apoptosis)	112.9

[a] The lowest BMDL median for GOBP represents the BMDL of the lowest GO term rank ordered by BMD Median.
[b] The 5th percentile BMDL Median for GOBP represents the 5th percentile of GO terms rank ordered by BMDL Median.
[c] The full MSigDB collection of gene sets.
[d] The Combined MSigDB Superset represents a consensus of multiple individual gene sets associated with a specific biological response. For this evaluation, cytotoxicity, apoptosis, inflammation, oxidative stress, and necrosis were prioritized.
The MSigDB and MSigDB superset results presented here are rank ordered by gene set-level BMDL Median and report the lowest and distribution-based 5th percentile gene set BMDL Medians for comparison.
For gene set accessions, the "GO" prefix refers to Gene Ontology accession identifiers, while the "M" prefix refers to MSigDB accession identifiers that were mapped via orthology from human gene sets since there are no native rat gene sets available in MSigDB.

Table_Apx C-6. BMD Modeling Results of Targeted Gene Sets Relating to Apical Effects from *p*-Dichlorobenzene Exposure (Apoptosis, Cytotoxicity, Inflammation, Oxidative Stress, Necrosis) Within Rat Kidney

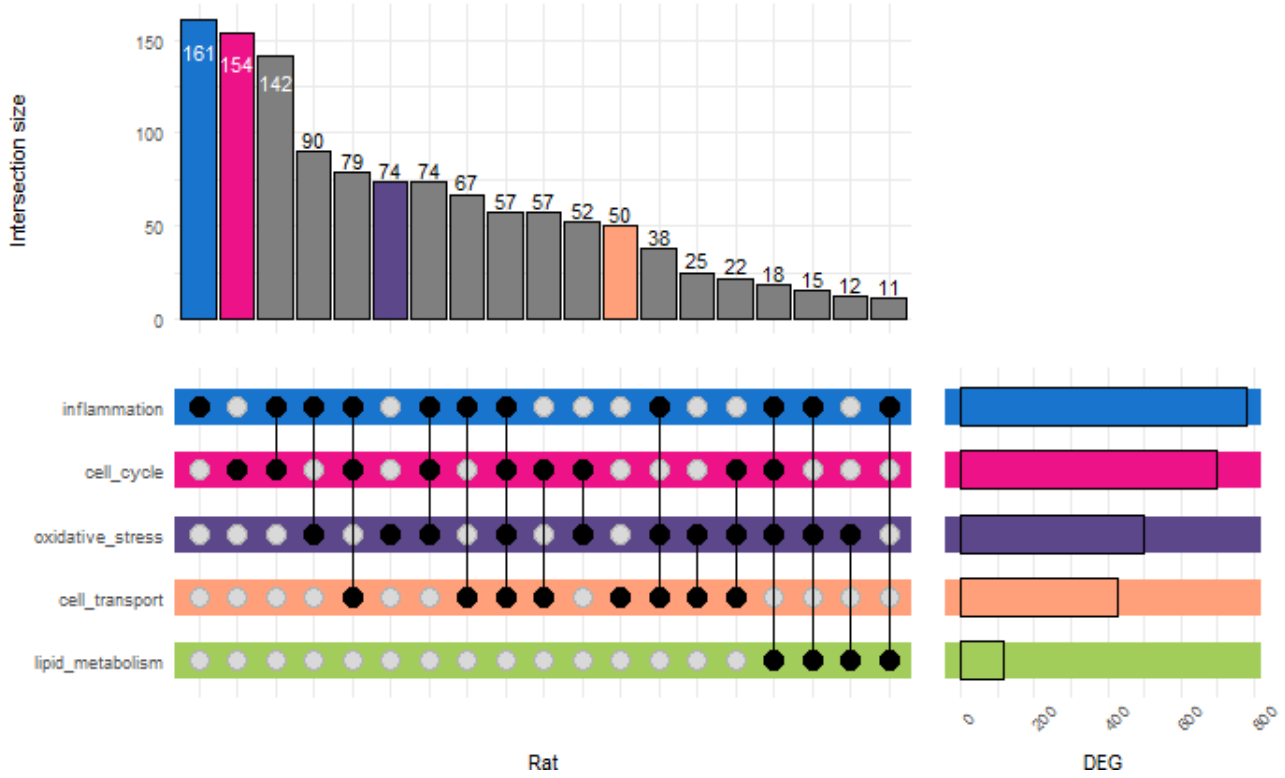
	Gene Set Accession	Lowest BMDL Median[a] (ppm)	5th Percentile BMDL Median[b] (ppm)
GOBP	GO:0030522	179	267.28
MSigDB[c]	M3533	62.74	209.75
MSigDB Combined Superset[d]	M14151	178.91 (apoptosis)	266.11

[a]The lowest BMDL median for GOBP represents the BMDL of the lowest GO term rank ordered by BMD Median.
[b]The 5th percentile BMDL Median for GOBP represents the 5th percentile of GO terms rank ordered by BMDL Median.
[c]The full MSigDB collection of gene sets.
[d]The Combined MSigDB Superset represents a consensus of multiple individual gene sets associated with a specific biological response. For this evaluation, cytotoxicity, apoptosis, inflammation, oxidative stress, and necrosis were prioritized.
The MSigDB and MSigDB superset results presented here are rank ordered by gene set-level BMDL Median and report the lowest and distribution-based 5th percentile gene set BMDL Medians for comparison.
For gene set accessions, the "GO" prefix refers to Gene Ontology accession identifiers, while the "M" prefix refers to MSigDB accession identifiers that were mapped via orthology from human gene sets since there are no native rat gene sets available in MSigDB.

C.2 Superset Expression Analysis for *p*-Dichlorobenzene

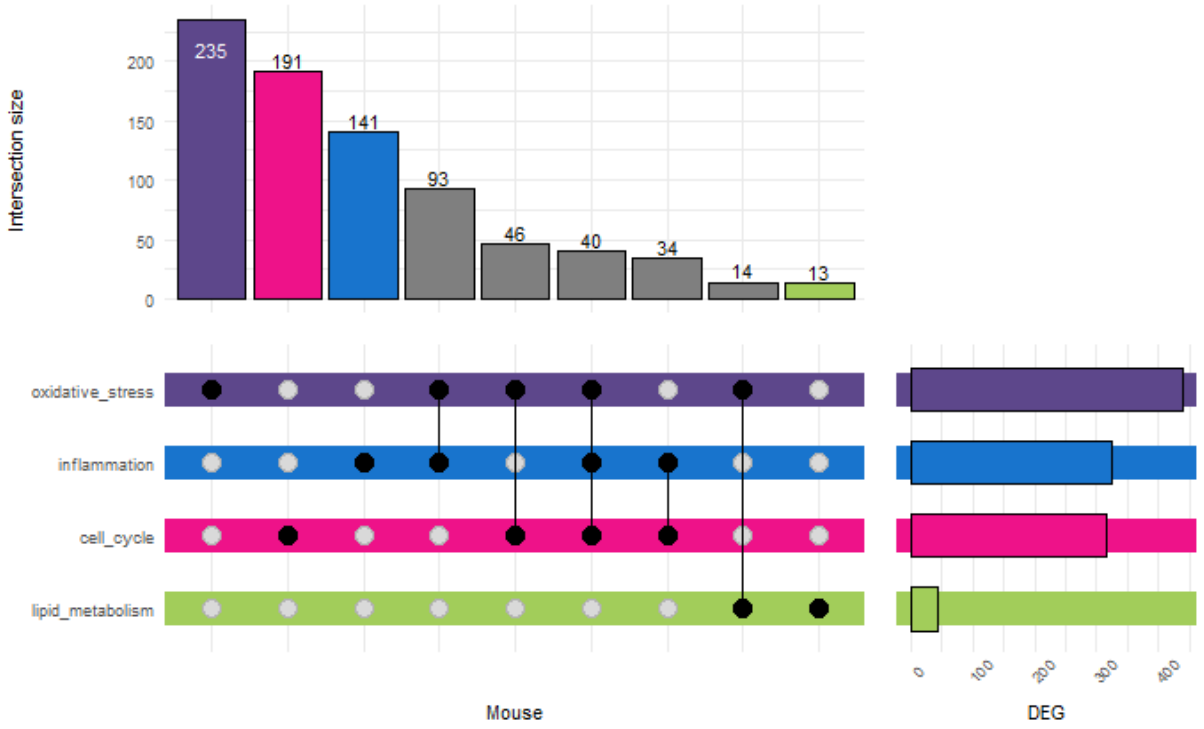
EPA identified an additional custom-curated superset terms for *p*-dichlorobenzene by qualitatively examining the most frequently disrupted pathway terms across the full MSigDB biological landscape.

These terms were compared with the apical effects described within the *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene* (U.S. EPA, 2026b), and it was determined that the predominant relevant pathways were related to inflammation, cell cycle, lipid metabolism, oxidative stress, and cell transport. Superset terms specific for a follow-up *p*-dichlorobenzene analysis were established using these five pathways; the terms were queried in the MSigDB library using the following search terms, respectively, to capture the broadest range of inclusive matches: "inflam," "cell cycle," "lipid met," "oxidative," and "cell trans." This custom-curated superset approach for *p*-dichlorobenzene was employed to develop a *p*-dichlorobenzene-specific gene signature that could be used to directly compare the pattern of gene expression deregulation between chemicals. Similar to the supersets for *o*-dichlorobenzene, there was overlap within the lists of genes that comprised of the *p*-dichlorobenzene supersets. Specifically, for rat genes Figure_Apx C-3, there were 1,258 total unique genes across these 5 supersets, with 161 unique to inflammation, 154 unique to cell cycle, 74 unique to oxidative stress, and 50 unique to cell transport. The remaining genes were shared by at least two unique supersets. For mouse genes (Figure_Apx C-4), there were 825 total unique genes across the 5 pathways, with 235 unique to oxidative stress, 191 unique to cell cycle, 141 unique to inflammation, and 13 unique to lipid metabolism. The remaining genes were shared by at least two unique supersets.



Figure_Apx C-3. UpSet Plot Showing the Overlap of Rat Genes Included as Inputs in BMD Modeling That Were Mapped to Each of the MSigDB Gene Set Terms in a Superset Using the Five Terms Determined from the Analysis of the *p*-Dichlorobenzene Transcriptomic Data: "Inflam," "Cell Cycle," "Lipid Met," "Oxidative," and "Cell Trans"

The UpSet plot shows "sets" of DEGs in the bottom-right corner, highlighting the total number of DEGs involved in each superset. The "intersections" of sets in the bar graph along the top show the 'exclusive intersection', which represents the total number of unique DEGs attributable to each superset or intersection of supersets. The black circles highlight which sets are included in each possible intersection across supersets. Only intersections with at least 10 members are shown.



Figure_Apx C-4. UpSet Plot Showing the Overlap of Mouse Genes Included as Inputs in BMD Modeling That Were Mapped to Each of the MSigDB Gene Set Terms in a Superset Using the Five Terms Determined from the Analysis of the *p*-Dichlorobenzene Transcriptomic Data: "Inflam," "Cell Cycle," "Lipid Met," "Oxidative," and "Cell Trans"

The UpSet plot shows "sets" of DEGs in the bottom-right corner, highlighting the total number of DEGs involved in each superset. The "intersections" of sets in the bar graph along the top show the 'exclusive intersection', which represents the total number of unique DEGs attributable to each superset or intersection of supersets. The black circles highlight which sets are included in each possible intersection across supersets. Only intersections with at least 10 members are shown.

C.3 tPOD Determination Analysis for *p*-Dichlorobenzene Using *p*-Dichlorobenzene Superset Terms

The MSigDB superset terms used for the previous analyses were expert-curated based on the *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene* (U.S. EPA, 2026a); it was plausible that the terms were not applicable to *p*-dichlorobenzene, which has different apical outcomes than *o*-dichlorobenzene (U.S. EPA, 2026b, d). To evaluate whether the updated list of MSigDB superset terms created based on the transcriptomic signatures observed following *p*-dichlorobenzene exposure and identified based on the *Draft Human Health and Environmental Hazard Assessment for p-Dichlorobenzene* (U.S. EPA, 2026b) were more applicable for *p*-dichlorobenzene outcomes, updated specific superset tPODs are reported below. Because the GOBP and MSigDB tPODs ($_{low}$ and $_{0.05}$) were unchanged by updated selection of MSigDB superset terms, only the updated superset tPODs (MSigDB SUPER$_{low}$ and MSigDB SUPER$_{0.05}$) are described in the text below. CAR signaling was additionally identified as a relevant term in the transcriptomic supersets but based on the conclusions of the *p*-dichlorobenzene report that CAR signaling resulting in rodent liver tumors is not a relevant mechanism in humans, the CAR term was removed from the superset term list.

The MSigDB SUPER$_{low}$ tPOD in the mouse liver of 13.41 ppm for the GOBP term "positive regulation of metaphase anaphase transition of cell cycle." The MSigDB SUPER$_{0.05}$ tPOD within the combined

MSigDB superset was 36.96 ppm. A full table of mouse liver BMD results for *p*-dichlorobenzene can be found in Table_Apx C-7, and accumulation plots for the combined MSigDB superset compared with the full landscape of gene sets in MSigDB are shown in Figure_Apx C-5 (MSigDB $SUPER_{low}$) and Figure_Apx C-6 (MSigDB $SUPER_{0.05}$).

The MSigDB $SUPER_{low}$ tPOD in the mouse lung of 2.67 ppm for the Reactome curated term "cell cycle." The MSigDB $SUPER_{0.05}$ tPOD within the combined MSigDB superset was 9.92 ppm. A full table of mouse lung BMD results for *p*-dichlorobenzene can be found in Table_Apx C-8, and accumulation plots for the combined MSigDB superset compared with the full landscape of gene sets in MSigDB are shown in Figure_Apx C-5 (MSigDB $SUPER_{low}$) and Figure_Apx C-6 (MSigDB $SUPER_{0.05}$).

The MSigDB $SUPER_{low}$ tPOD in the mouse kidney of 117.34 ppm for the GOBP term "oxidoreductase activity." The MSigDB $SUPER_{0.05}$ tPOD within the combined MSigDB superset was 117.34 ppm. A full table of mouse kidney BMD results for *p*-dichlorobenzene can be found in Table_Apx C-9, and accumulation plots for the combined MSigDB superset compared with the full landscape of gene sets in MSigDB are shown in Figure_Apx C-5 (MSigDB $SUPER_{low}$) and Figure_Apx C-6 (MSigDB $SUPER_{0.05}$).

Table_Apx C-7. BMD Modeling Results of Targeted Gene Sets Relating to Apical Effects from *p*-Dichlorobenzene Exposure (Cell Cycle, Cell Transport, Inflammation, Oxidative Stress, Lipid Metabolism) Within Mouse Liver

	Gene Set Accession	Lowest BMDL Median[a] (ppm)	5th Percentile BMDL Median[b] (ppm)
GOBP	GO:0048714	7.73	54.76
MSigDB[c]	GO:0048714	7.73	54.41
MSigDB Combined Superset[d]	MM10809	13.41 (cell cycle)	36.96

[a] The lowest BMDL median for GOBP represents the BMDL of the lowest GO term rank ordered by BMD Median.
[b] The 5th percentile BMDL Median for GOBP represents the 5th percentile of GO terms rank ordered by BMDL Median.
[c] The $MSigDB_{low}$ gene set, considering the full landscape of MSigDB gene sets.
[d] The Combined MSigDB Superset represents a consensus of multiple individual gene sets associated with a specific biological response. For this evaluation, cell cycle, cell transport, inflammation, oxidative stress, and lipid metabolism were prioritized.
The MSigDB and MSigDB superset results presented here are rank ordered by gene set-level BMDL Median and report the lowest and 5th percentile gene set BMDL Medians for comparison. For gene set accessions, the "GO" prefix refers to Gene Ontology accession identifiers, while the "MM" prefix refers to mouse MSigDB accession identifiers.

Table_Apx C-8. BMD Modeling Results of Targeted Gene Sets Relating to Apical Effects from *p*-Dichlorobenzene Exposure (Cell Cycle, Cell Transport, Inflammation, Oxidative Stress, Lipid Metabolism) Within Mouse Lung

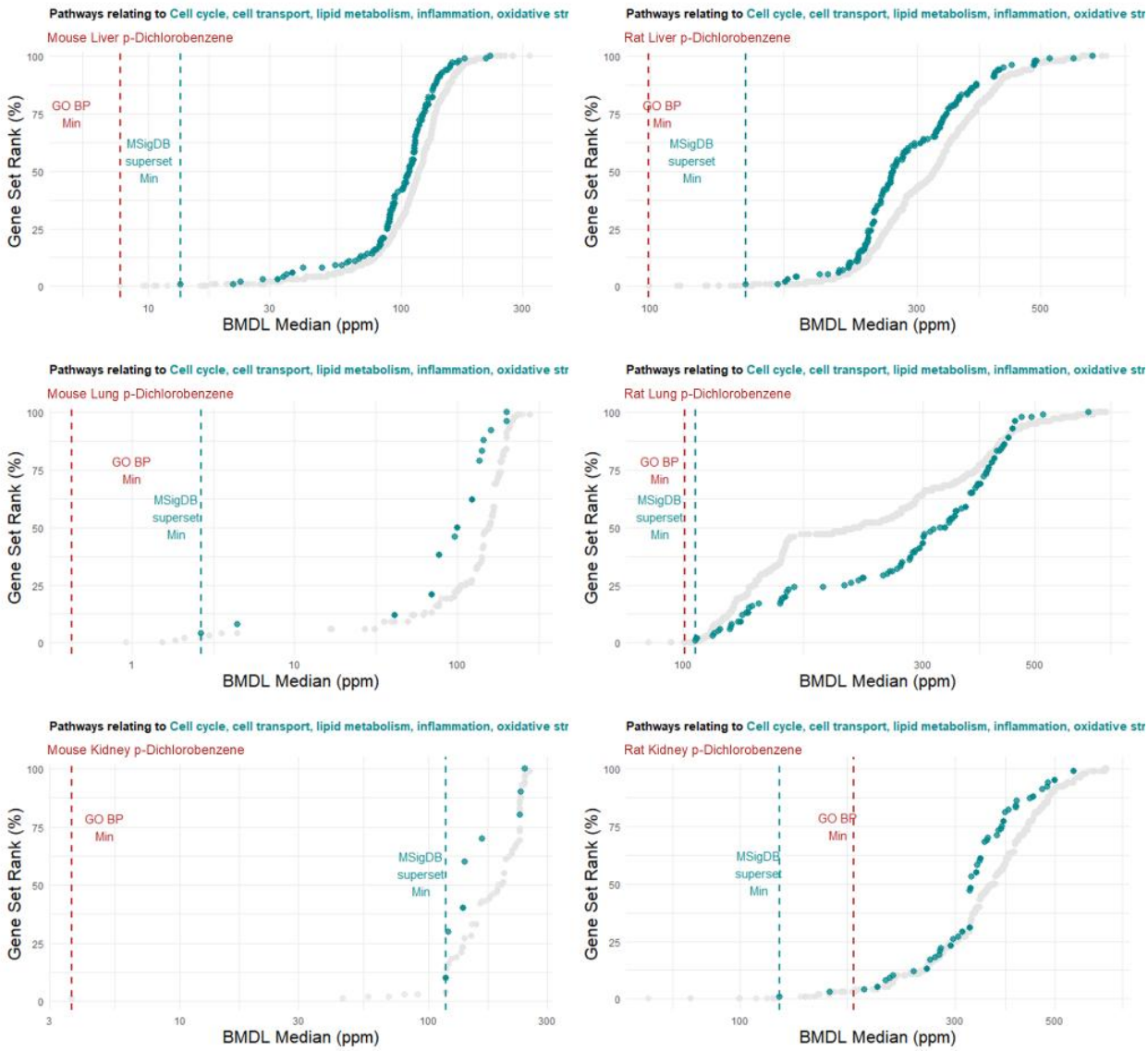
	Gene Set Accession	Lowest BMDL Median[a] (ppm)	5th Percentile BMDL Median[b] (ppm)
GOBP	GO:0071407	0.43	29.94
MSigDB[c]	GO:0046903	0.92	4.43
MSigDB Combined Superset[d]	MM14635	2.67 (cell cycle)	9.92

[a] The lowest BMDL median for GOBP represents the BMDL of the lowest GO term rank ordered by BMD Median.
[b] The 5th percentile BMDL Median for GOBP represents the 5th percentile of GO terms rank ordered by BMDL Median.
[c] The full $MSigDB_{low}$ gene set.
[d] The Combined MSigDB Superset represents a consensus of multiple individual gene sets associated with a specific biological response. For this evaluation, cell cycle, cell transport, inflammation, oxidative stress, and lipid metabolism were prioritized.
The MSigDB and MSigDB superset results presented here are rank ordered by gene set-level BMDL Median and report the lowest and 5th percentile gene set BMDL Medians for comparison. For gene set accessions, the "GO" prefix refers to Gene Ontology accession identifiers, while the "MM" prefix refers to mouse MSigDB accession identifiers.

Table_Apx C-9. BMD Modeling Results of Targeted Gene Sets Relating to Apical Effects from *p*-Dichlorobenzene Exposure (Cell Cycle, Cell Transport, Inflammation, Oxidative Stress, Lipid Metabolism) Within Mouse Kidney

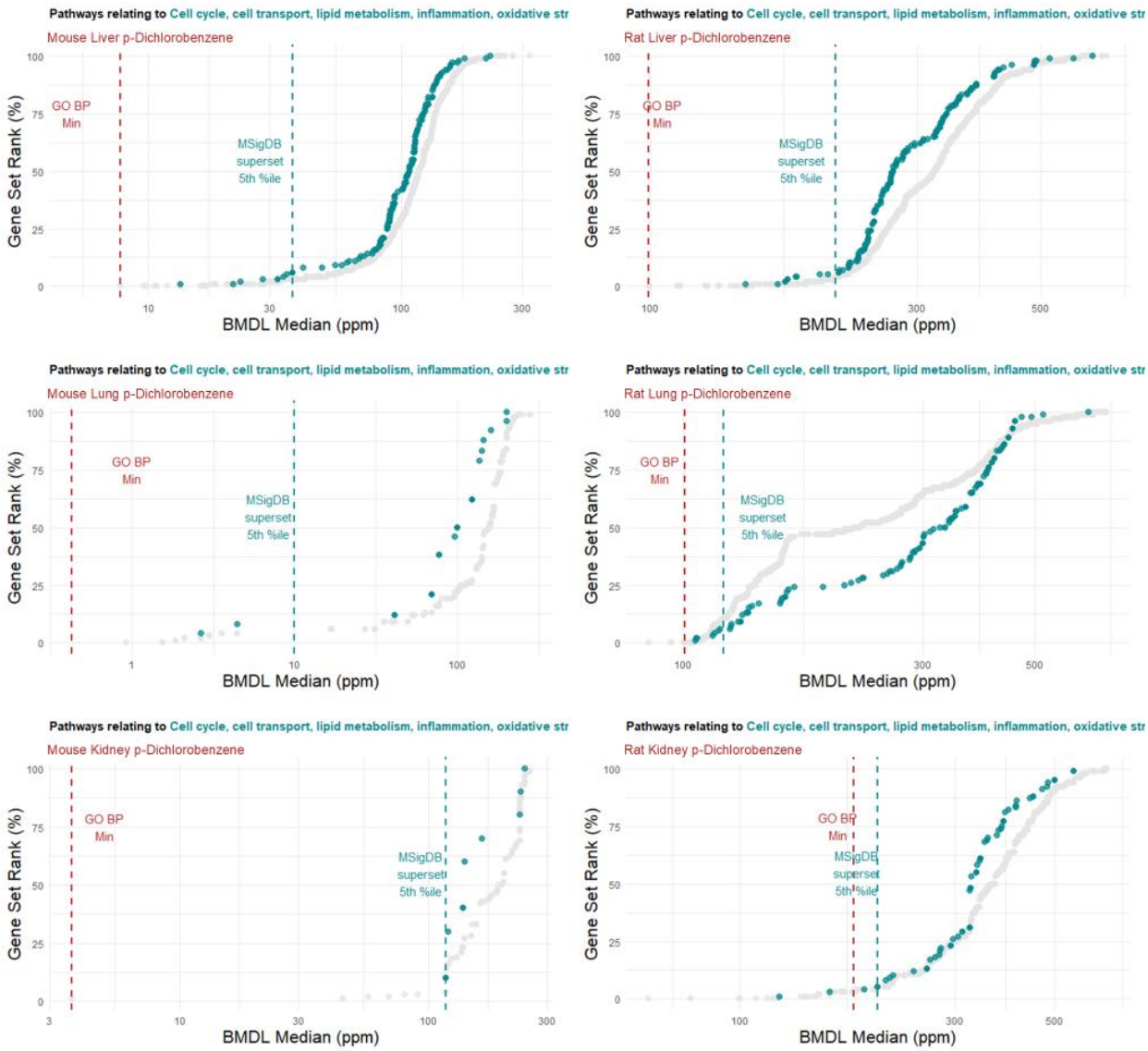
	Gene Set Accession	Lowest BMDL Median[a] (ppm)	5th Percentile BMDL Median[b] (ppm)
GOBP	GO:0006810	3.71	57.40
MSigDB[c]	GO:0015711	3.71	117.34
MSigDB Combined Superset[d]	MM13447, MM13477	117.34 (oxidative stress)	117.34

[a] The lowest BMDL median for GOBP represents the BMDL of the lowest GO term rank ordered by BMD Median.
[b] The 5th percentile BMDL Median for GOBP represents the 5th percentile of GO terms rank ordered by BMDL Median.
[c] The full $MSigDB_{low}$ gene set.
[d] The Combined MSigDB Superset represents a consensus of multiple individual gene sets associated with a specific biological response. For this evaluation, cell cycle, cell transport, inflammation, oxidative stress, and lipid metabolism were prioritized.
The MSigDB and MSigDB superset results presented here are rank ordered by gene set-level BMDL Median and report the lowest and 5th percentile gene set BMDL Medians for comparison. For gene set accessions, the "GO" prefix refers to Gene Ontology accession identifiers, while the "MM" prefix refers to mouse MSigDB accession identifiers.



Figure_Apx C-5. Mouse and Rat Liver, Lung, and Kidney Accumulation Plots Showing the Gene Set Ranks (%) for All Gene Sets in the MSigDB Landscape with Grayed Circles for *p*-Dichlorobenzene Exposure

The combined superset with any term from cell cycle, cell transport, inflammation, oxidative stress, lipid metabolism, and including at least three genes with BMD meeting all inclusion criteria, is shown against the landscape of all gene sets in color, with each subsequent plot highlighting a specific subset of those gene sets. The vertical dashed red line highlights the $GOBP_{low}$ tPOD, and the other highlights the MSigDB $SUPER_{low}$ tPOD fpr the combined MSigDB superset covering the terms "cell cycle," "cell trans," "inflam," "oxidative," and "lipid met."



Figure_Apx C-6. Mouse and Rat Liver, Lung, and Kidney Accumulation Plots Showing the Gene Set Ranks (%) for All Gene Sets in the MSigDB Landscape with Grayed Circles for *p*-Dichlorobenzene Exposure

The combined superset with any term from cell cycle, cell transport, inflammation, oxidative stress, lipid metabolism, and including at least three genes with BMD meeting all inclusion criteria, is shown against the landscape of all gene sets in color, with each subsequent plot highlighting a specific subset of those gene sets. The vertical dashed red line highlights the $GOBP_{low}$ tPOD, and the other highlights the MSigDB $SUPER_{0.05}$ tPOD for the combined MSigDB superset covering the terms "cell cycle," "cell trans," "inflam," "oxidative," and "lipid met."

The MSigDB $SUPER_{low}$ tPOD in the rat liver of 148.23 ppm for the GOBP term "metaphase anaphase transition of meiotic cell cycle." The MSigDB $SUPER_{0.05}$ tPOD within the combined MSigDB superset was 214.55 ppm. A full table of mouse liver BMD results for *p*-dichlorobenzene can be found in Table_Apx C-10, and accumulation plots for the combined MSigDB superset compared with the full landscape of gene sets in MSigDB are shown in Figure_Apx C-5 (MSigDB $SUPER_{low}$) and Figure_Apx C-6 (MSigDB $SUPER_{0.05}$). It is notable that inclusion of CAR as a superset term would have affected

the combined MSigDB superset results by changing the $MSigDB_{low}$ tPOD to 111.57 ppm and the $MSigDB_{0.05}$ tPOD to 206.38 ppm.

The MSigDB $SUPER_{low}$ tPOD in the rat lung of 105.88 ppm for the term "microbody." The MSigDB $SUPER_{0.05}$ tPOD within the combined MSigDB superset was 120.24 ppm. A full table of mouse lung BMD results for *p*-dichlorobenzene can be found in Table_Apx C-11, and accumulation plots for the combined MSigDB superset compared with the full landscape of gene sets in MSigDB are shown in Figure_Apx C-5 (MSigDB $SUPER_{low}$) and Figure_Apx C-6 (MSigDB $SUPER_{0.05}$). It is notable that inclusion of CAR as a superset term would have affected the combined MSigDB superset results by changing the $MSigDB_{0.05}$ tPOD to 120.62 ppm.

The MSigDB $SUPER_{low}$ tPOD in the rat kidney of 122.31 ppm for the Whitfield curated term "cell cycle M to G1." The MSigDB $SUPER_{0.05}$ tPOD within the combined MSigDB superset was 202.26 ppm. A full table of mouse kidney BMD results for *p*-dichlorobenzene can be found in Table_Apx C-12, and accumulation plots for the combined MSigDB superset compared with the full landscape of gene sets in MSigDB are shown in Figure_Apx C-5 (MSigDB $SUPER_{low}$) and Figure_Apx C-6 (MSigDB $SUPER_{0.05}$).

Table_Apx C-10. BMD Modeling Results of Targeted Gene Sets Relating to Apical Effects from *p*-Dichlorobenzene Exposure (Cell Cycle, Cell Transport, Inflammation, Oxidative Stress, Lipid Metabolism) Within Rat Liver

	Gene Set Accession	Lowest BMDL Median[a] (ppm)	5th Percentile BMDL Median[b] (ppm)
GOBP	GO:0098656	99.3	228.82
MSigDB[c]	GO:0006820	99.34	222.62
MSigDB Combined Superset[d]	M46805	148.23 (cell cycle)	214.55

[a] The lowest BMDL median for GOBP represents the BMDL of the lowest GO term rank ordered by BMD Median.
[b] The 5th percentile BMDL Median for GOBP represents the 5th percentile of GO terms rank ordered by BMDL Median.
[c] The full $MSigDB_{low}$ gene set.
[d] The Combined MSigDB Superset represents a consensus of multiple individual gene sets associated with a specific biological response. For this evaluation, cell cycle, cell transport, inflammation, oxidative stress, and lipid metabolism were prioritized.
The MSigDB and MSigDB superset results presented here are rank ordered by gene set-level BMDL Median and report the lowest and 5th percentile gene set BMDL Medians for comparison. For gene set accessions, the "GO" prefix refers to Gene Ontology accession identifiers, while the "M" prefix refers to MSigDB accession identifiers that were mapped via orthology from human gene sets since there are no native rat gene sets available in MSigDB.

Table_Apx C-11. BMD Modeling Results of Targeted Gene Sets Relating to Apical Effects from *p*-Dichlorobenzene Exposure (Cell Cycle, Cell Transport, Inflammation, Oxidative Stress, Lipid Metabolism) Within Rat Lung

	Gene Set Accession	Lowest BMDL Median[a] (ppm)	5th Percentile BMDL Median[b] (ppm)
GOBP	GO:0006486	101	120.43
MSigDB[c]	M6958	85.56	113.96
MSigDB Combined Superset[d]	M17410	105.88 (oxidative stress)	120.24

[a] The lowest BMDL median for GOBP represents the BMDL of the lowest GO term rank ordered by BMD Median.
[b] The 5th percentile BMDL Median for GOBP represents the 5th percentile of GO terms rank ordered by BMDL Median.
[c] The full $MSigDB_{low}$ gene set.
[d] The Combined MSigDB Superset represents a consensus of multiple individual gene sets associated with a specific biological response. For this evaluation, cell cycle, cell transport, inflammation, oxidative stress, and lipid metabolism were prioritized.
The MSigDB and MSigDB superset results presented here are rank ordered by gene set-level BMDL Median and report the lowest and 5th percentile gene set BMDL Medians for comparison. For gene set accessions, the "GO" prefix refers to Gene Ontology accession identifiers, while the "M" prefix refers to MSigDB accession identifiers that were mapped via orthology from human gene sets since there are no native rat gene sets available in MSigDB.

Table_Apx C-12. BMD Modeling Results of Targeted Gene Sets Relating to Apical Effects from p-Dichlorobenzene Exposure (Cell Cycle, Cell Transport, Inflammation, Oxidative Stress, Lipid Metabolism) Within Rat Kidney

	Gene Set Accession	Lowest BMDL Median[a] (ppm)	5th Percentile BMDL Median[b] (ppm)
GOBP	GO:0030522	179	267.28
MSigDB[c]	M3533	62.74	209.75
MSigDB Combined Superset[d]	M2078	122.31 (cell cycle)	202.26

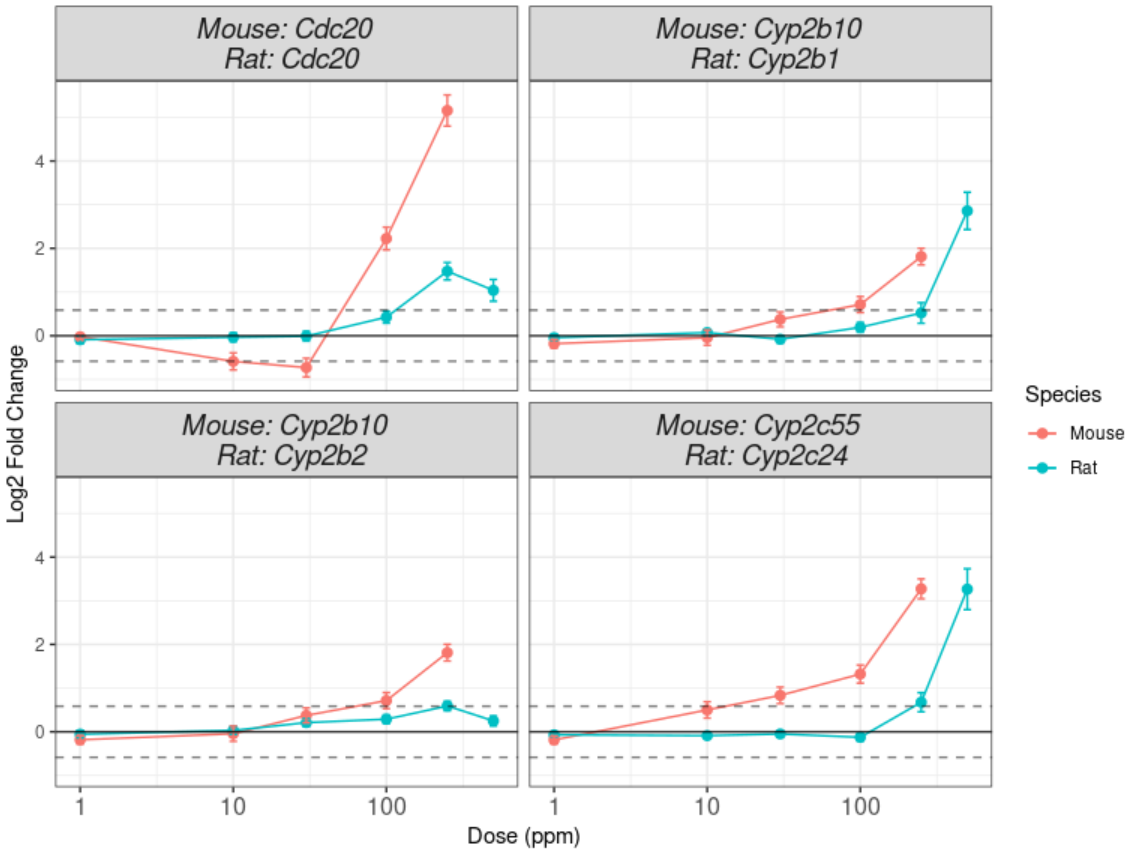
[a] The lowest BMDL median for GOBP represents the BMDL of the lowest GO term rank ordered by BMD Median.
[b]The 5th percentile BMDL Median for GOBP represents the 5th percentile of GO terms rank ordered by BMDL Median.
[c] The full $MSigDB_{low}$ gene set.
[d] The Combined MSigDB Superset represents a consensus of multiple individual gene sets associated with a specific biological response. For this evaluation, cell cycle, cell transport, inflammation, oxidative stress, and lipid metabolism were prioritized.
The MSigDB and MSigDB superset results presented here are rank ordered by gene set-level BMDL Median and report the lowest and 5th percentile gene set BMDL Medians for comparison. For gene set accessions, the "GO" prefix refers to Gene Ontology accession identifiers, while the "M" prefix refers to MSigDB accession identifiers that were mapped via orthology from human gene sets since there are no native rat gene sets available in MSigDB.

Appendix D *o*-DICHLOROBENZENE CAR GENE BIOMARKER ANALYSIS

The EPA *Draft Human Health and Environmental Hazard Assessment for o-Dichlorobenzene* (U.S. EPA, 2026a) did not identify an adequate chronic assay to assess carcinogenicity, and thus evidence for carcinogenicity was evaluated using early endpoints induced by *o*-dichlorobenzene or orthologs by the ReCAAP framework (U.S. EPA, 2026a, c). This framework systematically utilizes available information about endpoints linked to carcinogenesis. Given that *p*-dichlorobenzene induces mouse liver tumors, endpoints for key events in rodent liver cancer MOAs were evaluated. There was a paucity of information available to assess whether any of the MOAs were activated by *o*-dichlorobenzene. However, the 5-day *in vivo* transcriptomic study provided EPA with the opportunity to assess whether the MIEs of rodent liver MOA proposed *for p*-dichlorobenzene is also observed in rodent livers exposed to *o*-dichlorobenzene. For completeness analysis, this Appendix details the results of the same CAR biomarker gene analysis performed in Section 4.3 but applied to *o*-dichlorobenzene.

D.1 Evaluation of CAR Biomarker Gene Set for *o*-Dichlorobenzene

Exposure to *o*-dichlorobenzene resulted in large fold change increases across all genes in the CAR biomarker panel for both rat and mouse liver (Figure_Apx D-1). For all genes, the $\log_2$ fold change dose-response in mouse was left-shifted compared to rats at equivalent doses, indicating that mouse livers exposed to *o*-dichlorobenzene were more sensitive than rats, and the overall magnitude of change in expression in the CAR biomarker gene set in mice was more pronounced. Interestingly for rat liver, the magnitude of the dose-response in expression changes between *Cyp2b1* and *Cyp2b2* was different, with *Cyp2b2* not showing strong dose-responsive effects. This may be attributable to lower basal expression of *Cyp2b1* or via differential regulation of the two isoforms. The BMD modeling results for the CAR biomarker gene set that met the criteria for BMD modeling are presented in Table_Apx D-1. The BMD and BMDL for *Cyp2b1* and *Cyp2c24* were much lower in mice compared to rats. In contrast, the BMD and BMDL values for *Cdc20* and *Cyp2b2* were lower in rats compared to mice based on nominal exposure concentration; however, the overall magnitude in the dose-response was more robust in mice than in rats for these genes. Taken together, it appears that the induction of the CAR biomarker gene set was more pronounced in mice than in rats.



Figure_Apx D-1. Dose-Response of the Log$_2$ Fold Change Expression of the CAR Biomarker Gene Set in Mouse and Rat Livers After *o*-Dichlorobenzene Exposure

Error bars denote the standard error in estimated log$_2$ fold change values Horizontal dashed lines indicate an absolute log$_2$ fold change of 1.5 to designate a significant increase or decrease in fold change compared to vehicle control samples. Note, the log$_2$ fold change expression for *Cyp2b1* and *Cyp2b2* use the same data from the mouse *Cyp2b10* ortholog.

Table_Apx D-1. BMD Modeling Results of the CAR Biomarker Gene Set in Mouse or Rat Livers Exposed to *o*-Dichlorobenzene

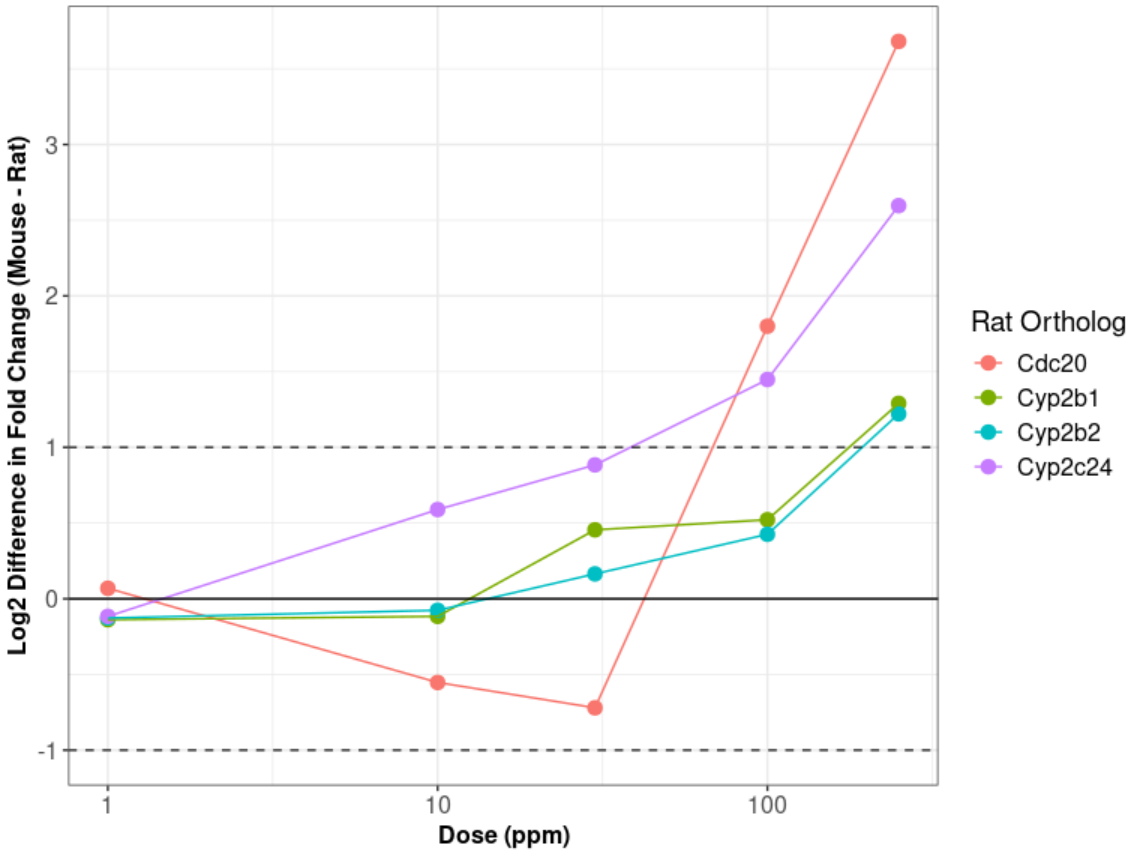
Gene Symbol	Mouse BMD (ppm)	Mouse BMDL (ppm)	Rat BMD (ppm)	Rat BMDL (ppm)
Cdc20	90.64	53.23	51.17	39.29
Cyp2b1[a]	66.05	52.26	174.28	136.72
Cyp2b2[a]	66.05	52.26	61.53	46.00
Cyp2c24[b]	28.41	14.34	246.90	217.52

[a] Orthologous to mouse *Cyp2b10*
[b] Orthologous to mouse *Cyp2c55*
Note: *Cyp2b1* and *Cyp2b2* BMD modeling results for mouse are identical since they were calculated from the mouse ortholog, *Cyp2b10*

D.2 Species Comparison of CAR Biomarker Gene Set for *o*-Dichlorobenzene

The highest comparable dose that was exposed to both species was 250 ppm. *o*-dichlorobenzene exposure at 250 ppm in mouse resulted in log$_2$ fold increases of 5.16 (*Cdc20*), 1.81 (*Cyp2b10*), and 3.27

(*Cyp2c55*). In contrast, the 250 ppm dose group in rat only elicited fold increases of 1.48 (*Cdc20*), 0.52 (*Cyp2b1*; functionally orthologous to mouse *Cyp2b10*), 0.59 (*Cyp2b2*; functionally orthologous to mouse *Cyp2b10*), and 0.68 (*Cyp2c24*; functionally orthologous to mouse *Cyp2c55*). Figure_Apx D-2 examines the overall $\log_2$ fold change difference between equivalent doses of *o*-dichlorobenzene in liver between mouse and rat. *Cdc20* (functionally orthologous to mouse *Cdc20*) had the largest $\log_2$ fold change difference at the highest equivalent dose of 250 ppm, with mouse having 3.68 times higher $\log_2$ fold change expression compared to rat. *Cyp2c24* (functionally orthologous to mouse *Cyp2c55*) had the second largest $\log_2$ fold change difference, with mouse having 2.60 times higher $\log_2$ fold change expression compared to rat. Similarly, the $\log_2$ expression of *Cyp2b1* and *Cypb2b2* (functionally orthologous to mouse *Cyp2b10*) was 1.29 and 1.22 times higher than rat at 250 ppm exposure in liver, respectively.



Figure_Apx D-2. Species Comparison of Rat Orthologous CAR Biomarker Gene Set After *o*-Dichlorobenzene Exposure in Liver

Dashed horizontal lines indicate an absolute $\log_2$ fold change difference between mouse and rat of 1 (arithmetic 2-fold increase or decrease compared to mouse). Note, the comparison of *Cyp2b1* and *Cyp2b2* use the same data from the mouse *Cyp2b10* ortholog in the $\log_2$ difference in fold change calculation.