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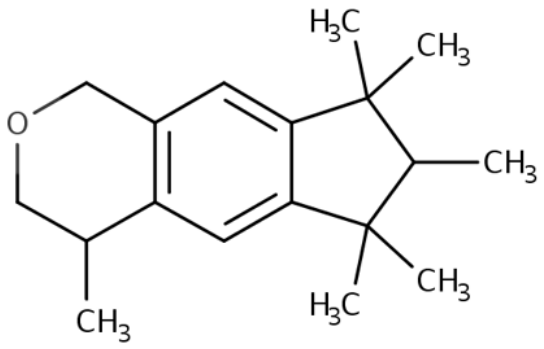
Draft Human Health and Environmental Hazard Assessment for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)

Technical Support Document for the Draft Risk Evaluation

CASRN 1222-05-5



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KEY ABBREVIATIONS AND ACRONYMS

ADME	Absorption, distribution, metabolism and excretion
AR	Androgen receptor
BMD	Benchmark dose
CASRN	Chemical Abstracts Service Registry Number
COC	Concentration of concern
DEP	Diethyl phthalate
DER	Data evaluation record

dw	Dry weight
ECB	European Chemicals Bureau
ECHA	European Chemicals Agency
EPA	Environmental Protection Agency (U.S.)
ER	Estrogen receptor
EU	European Union
EOGRT	Extended one-generation reproductive toxicity (OECD)
FSH	Follicle stimulating hormone
GD	Gestation day
GHS	Globally Harmonized System
HC05	Hazardous concentration for 5% of species (protecting 95% of species)
HEC	Human equivalent concentration
HED	Human equivalent dose
HRIPT	Human repeated insult patch test
HV	Hazard value
IP (or I.P.)	Intraperitoneal
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IRIS	Integrated Risk Information System (U.S.)
LC50	Lethal concentration at which 50% of test organisms die
LD	Lactation day
LD50	Lethal dose at which 50% of test organisms die
LH	Luteinizing hormone
LLNA	Local lymph node assay
LOAEL	Lowest-observed-adverse-effect level
LOEC	Lowest-observed-effect concentration
LOEL	Lowest-observed-effect level
lw	lipid weight
MOA	Mode of action
MOE	Margin of exposure
NICNAS	National Industrial Chemicals Notification and Assessment Scheme
NAM	New Approach Method
NOAEL	No-observed-adverse-effect level
NOEC	No-observed-effect concentration
NOEL	No-observed-effect level
OCSPP	Office of Chemical Safety and Pollution Prevention (EPA)
OECD	Organisation for Economic Co-operation and Development
OPP	Office of Pesticide Programs (EPA)
OPPT	Office of Pollution Prevention and Toxics (EPA)
PBPK	Physiologically based pharmacokinetic
PESS	Potentially exposed or susceptible subpopulations
PND	Postnatal day
POD	Point of departure
PR	Progesterone receptor
QSAR	Quantitative structure-activity relationship (model)
RAPD	Random amplified polymorphic DNA
RIFM	Research Institute for Fragrance Materials
SACC	Science Advisory Committee on Chemicals
SD	Sprague-Dawley (rats)
SDA 39C	Specially Denatured Alcohol 39C

SSD	Species sensitivity distribution
T4	Thyroxine
TSCA	Toxic Substances Control Act
TSH	Thyroid stimulating hormone
UF	Uncertainty factor
U.S.	United States

SUMMARY

This technical support document (TSD) accompanies the Toxic Substances Control Act (TSCA) *Draft Risk Evaluation for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (U.S. EPA, 2026i) (see also public docket, EPA-HQ-OPPT-2018-0430). This TSD describes the use of reasonably available information to evaluate the cancer and non-cancer human health hazards as well as the environmental health hazards associated with exposure to HHCB and the points of departure (PODs) to be used to estimate risks from HHCB exposures in the draft risk evaluation of HHCB.

Human Health Hazard Assessment

HHCB exposure can occur via inhalation, ingestion, or dermal contact. Therefore, the U.S. Environmental Protection Agency (EPA or the Agency) characterized the cancer and non-cancer hazards of HHCB for the oral, inhalation, and dermal routes of exposure and derived PODs as appropriate.

Non-Cancer Hazard Assessment: EPA focused on the endpoints and studies considered for deriving non-cancer hazard values in previous assessments by EPA (OCSPP, 2014) and the European Chemicals Bureau (ECB) (ECB, 2008a, b). These existing assessments derived non-cancer hazard values based on developmental toxicity and noted that this endpoint was uncertain given conflicting results in a small number of studies and given the lack of multigenerational reproductive toxicity studies at the time. Since these assessments were completed, an OECD (Organisation for Economic Co-operation and Development) 443 extended one-generation reproductive toxicity (EOGRT) study, a modified OECD 421 reproductive/developmental toxicity screening test, an OECD 414 prenatal developmental toxicity study in rabbits, and two non-guideline studies assessing reproductive toxicity in male rats became available to EPA (IFF, Date Unknown-b; Li and Wang, 2023; Li et al., 2023; IFF, 2021, 2020a). The Agency evaluated the data on non-cancer hazards associated with HHCB and determined that developmental and reproductive toxicity was the only hazard outcome appropriate for dose-response analysis.

HHCB, along with common mixtures of HHCB and other compounds, has been extensively evaluated for dermal absorption and dermal hazards in previous assessments. EPA considered the available studies on dermal absorption, acute and subchronic systemic dermal toxicity, dermal irritation, and dermal sensitization and determined that no dermal hazards were observed for HHCB at concentrations below or above those that are relevant to human exposure. This is due in part to HHCB's lipophilicity (discussed in Section 2.3 of the *Draft Physical Chemistry, Fate and Transport, Environmental Release, and Environmental Exposure Assessment for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (U.S. EPA, 2026f)), which leads to retention on or within the skin and minimal entry into systemic circulation. Therefore, only oral and inhalation hazard values were derived.

EPA considered the available acute oral, inhalation, and dermal toxicity studies as well as EOGRT and developmental toxicity studies and did not identify any health effects that were relevant for setting an acute POD. The Agency did not derive a hazard value for acute exposures for any route because the available evidence indicates it is unlikely that any adverse effects will result following a single exposure at concentrations relevant to human exposures. Additionally, the POD for repeated exposures is expected to be protective of any potential acute hazard.

For intermediate and chronic durations of oral exposure, EPA is proposing a POD of 30 mg/kg-day (human equivalent dose [HED] of 7.09 mg/kg-day) based on decreased offspring body weight to estimate non-cancer risks from oral exposure to HHCB. This POD was derived from benchmark dose

modeling of F1 offspring body weight data from PND (postnatal day) 1 through PND 21, at sexual maturation, and in adulthood from the EOGRT study in rats (IFF, 2021). This POD is protective of other effects identified in this study (delayed preputial separation in F1 offspring and decreased anogenital distance in F2 offspring) and is protective of maternal toxicity noted in other studies. The proposed POD is also protective of potentially exposed or susceptible subpopulations (PESS), including sensitive lifestages (pregnant women, infants, children, and adolescents) and will be protective of potential exposures to HHCB via cord blood and breastmilk. EPA has performed ¾-body weight scaling to yield the HED and is applying the animal to human uncertainty factor (*i.e.,* interspecies uncertainty factor; UF_A) of 3× and the human variability uncertainty factor (*i.e.,* intraspecies uncertainty factor; UF_H) of 10×. Thus, a total UF of 30× is applied for use as the benchmark MOE. Overall, based on the strengths, limitations, and uncertainties, EPA has robust overall confidence in the proposed (non-cancer) POD. This POD will be used to characterize risk from exposure to HHCB for intermediate and chronic oral exposure scenarios.

No data are available for the inhalation route that are suitable for deriving route-specific PODs. Therefore, EPA is using the intermediate/chronic oral POD to evaluate risks from inhalation exposure to HHCB. For the inhalation route, the Agency is extrapolating the oral HED to an inhalation human equivalent concentration (HEC) per EPA's *Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry* (U.S. EPA, 1994) using the updated human body weight and breathing rate relevant to continuous exposure of an individual at rest provided in EPA's *Exposure Factors Handbook* (U.S. EPA, 2011b). The oral HED and inhalation HEC values selected by EPA to estimate non-cancer risk from intermediate and chronic exposure to HHCB are summarized in Section 2.7.

Cancer Hazard Assessment: Previous assessments did not evaluate cancer risk because no cancer bioassays were available for HHCB. EPA did not identify any new cancer bioassays published since these assessments through the systematic review process. The Agency used elements of the Rethinking Chronic Toxicity and Carcinogenicity Assessment for Agrochemicals Project, or the ReCAAP weight of evidence framework as an organizational tool to evaluate the extent to which the lack of carcinogenicity studies imparts significant uncertainty on the human health risk assessments for HHCB (Hilton et al., 2022). Specifically, EPA evaluated the weight of evidence from physical and chemical properties, toxicokinetics, acute toxicity, subchronic toxicity, genotoxicity, hormone perturbation, immune system perturbation, mechanistic studies to support cancer MOA, and chronic toxicity. From these lines of evidence, EPA determined that HHCB has a low potential for bioaccumulation in human tissues; produces no acute toxicity, irritation, or sensitization; causes no adverse organ weight changes and no pre-neoplastic lesions after repeated subchronic exposure; is not genotoxic; is not an endocrine disruptor *in vivo*; and is not toxic to the immune system. EPA concluded that the lack of carcinogenicity bioassays for HHCB does not suggest that there are significant remaining scientific uncertainties in the qualitative and quantitative risk characterization for this chemical; The non-cancer POD that was selected for characterizing risk from intermediate and chronic exposure to HHCB is health protective. Therefore, EPA did not conduct a dose-response assessment for cancer and did not quantitatively evaluate HHCB for carcinogenic risk to human health.

Environmental Hazard Assessment

EPA considered all reasonably available information identified through the systematic review process to characterize environmental hazard endpoints for HHCB. After evaluating the reasonably available information, EPA concluded that HHCB is toxic to aquatic organisms (U.S. EPA, 2012b) and derived concentrations of concern (COCs) through acute and chronic HHCB exposures in water and sediment

(Table S-1). EPA also derived hazard thresholds for dietary HHCB exposure to terrestrial mammals and soil HHCB exposure to terrestrial invertebrates and plants.

Table S-1. Environmental Hazard Summary for HHCB

Receptor Group	Exposure Duration and Organism Hazard	Hazard Threshold (COC or HV)	Assessment Medium	Citation (Study Quality)
Aquatic animals (Section 3.2.1)	Acute exposure resulting in aquatic vertebrate and invertebrate species mortality	42.3 µg/L	Water column	From SSD
Aquatic vertebrates (Section 3.2.2)	Chronic exposure to aquatic vertebrate species (54% reduction in fish [*Pimphales promelas*] growth over 32 days)	9.8 µg/L	Water column	(Croudace et al., 1997) (High)
Sediment-dwelling invertebrates (Section 3.2.4)	Chronic exposure to sediment-dwelling animal species (49% reduction in *Lumbriculus variegatus* reproduction over 28 days)	2.4 mg/kg dw	Sediment	(IFF, Date Unknown-a) (High)
Terrestrial vertebrates (Section 3.3.1)	Chronic dietary exposure to terrestrial mammals (15% lower pup weight over 2 generations)	35.0 mg/kg/day	Diet	(IFF, 2021) (Acceptable/ Guideline)
Terrestrial invertebrates (Section 3.3.2)	Chronic exposure to soil invertebrates (30% lower earthworm [*Eisenia fetida*] reproduction over 28 days)	38.7 mg/kg	Soil	(Chen et al., 2011a) (High)
Terrestrial plants (Section 3.3.3)	Chronic exposure in soil to plants (50% lower rapeseed [*Brassica napus*] biomass over 21 days)	3.55 mg/kg	Soil	(IFF, 2019) (High)
COC = concentration of concern; dw = dry weight; HV = hazard value; SSD = species sensitivity distribution				

The HHCB hazard thresholds were within the same order of magnitude as thresholds derived in previous HHCB environmental hazard assessments (OCSPP, 2014; ECB, 2008b). EPA has robust confidence in the quality, consistency, strength and precision, and relevance of the studies used in determining these hazard thresholds.

1 INTRODUCTION

This TSD, also called the "draft human health and environmental hazard assessment," accompanies the Toxic Substances Control Act (TSCA) *Draft Risk Evaluation for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (also called the "draft HHCB risk evaluation") (U.S. EPA, 2026i).

A basic diagram of the HHCB risk evaluation and assessments/TSDs is provided in Figure 1-1. This draft TSD is shaded blue and focuses on human health (Section 2) and environmental (Section 3) hazard assessments for HHCB. The human health hazard assessment is organized as follows: Section 2.1 presents EPA's approach and methodology for the human health hazard assessment, including refinement of systematic review processes. The toxicokinetics of HHCB are discussed in Section 2.2. The following sections summarize the non-cancer (Section 2.3) and cancer (Section 2.5) human health hazards associated with exposure to HHCB. The non-cancer dose-response analysis is in Section 2.4. An analysis of PESS along with considerations for aggregate exposure is described in Section 2.6. Finally, the draft human hazard values to be used for human health risk estimates are summarized in Section 2.7. The environmental hazard assessment includes EPA's approach and methodology for the environmental hazard assessment (Section 3.1). Descriptions of the hazards, hazard thresholds, and weight of scientific evidence for the hazard thresholds of HHCB to aquatic organisms and terrestrial organisms are described in Sections 3.2 and 3.3, respectively. Lastly, Section 3.4 provides summaries and conclusions of the environmental hazard thresholds and the weight of scientific evidence for the environmental hazard assessment.

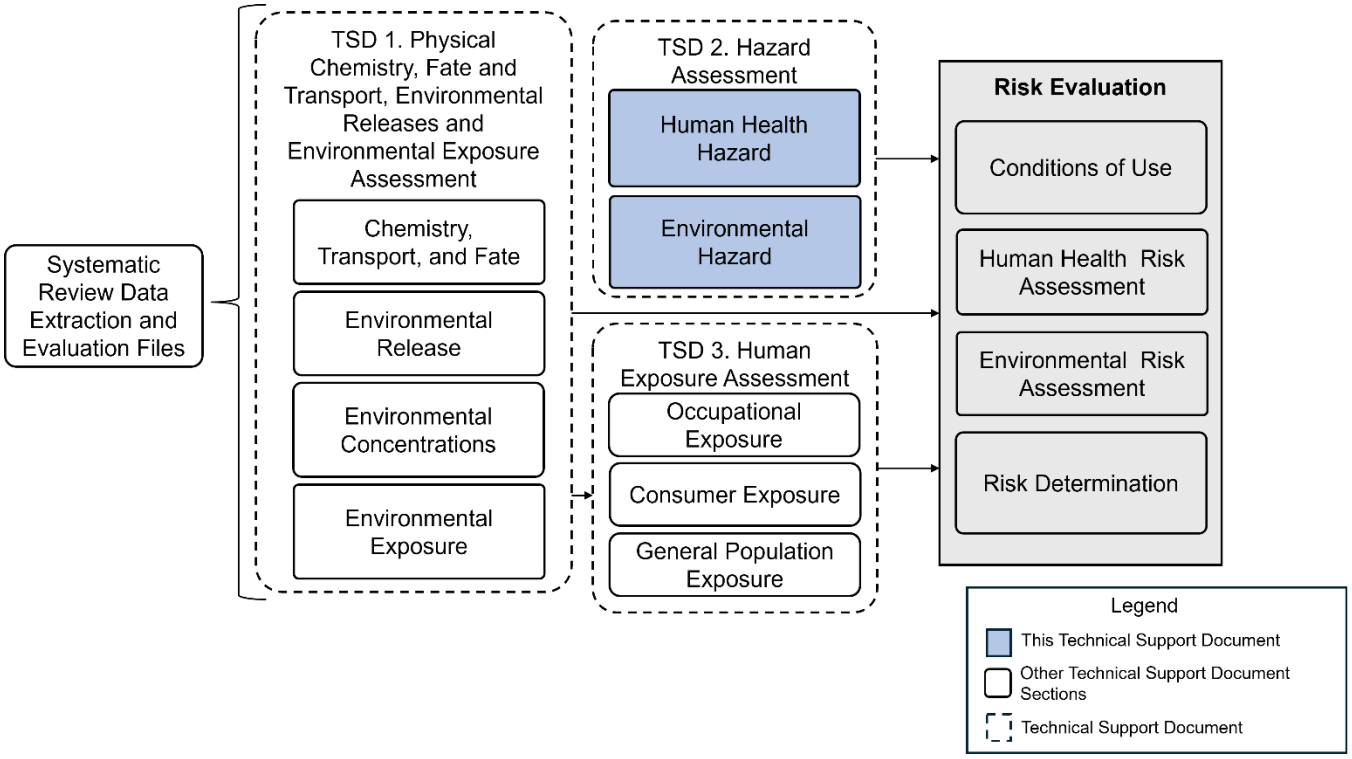

Figure 1-1. Document Map Summary for the Draft HHCB Risk Evaluation

2 HUMAN HEALTH HAZARD ASSESSMENT

2.1 Approach and Methodology

EPA's Office of Pollution Prevention and Toxics (OPPT) utilized systematic review processes to search, screen, evaluate, extract, and integrate reasonably available information to make conclusions about relevant adverse health effects from HHCB exposure. Following evidence integration, EPA performed dose-response analysis to derive hazard values for use in risk characterization. The Agency then evaluated the weight of scientific evidence for each aspect of the assessment and determined overall confidence ratings for each critical hazard outcome. The generalized process for conducting human health assessments under TSCA is presented below in Figure 2-1.

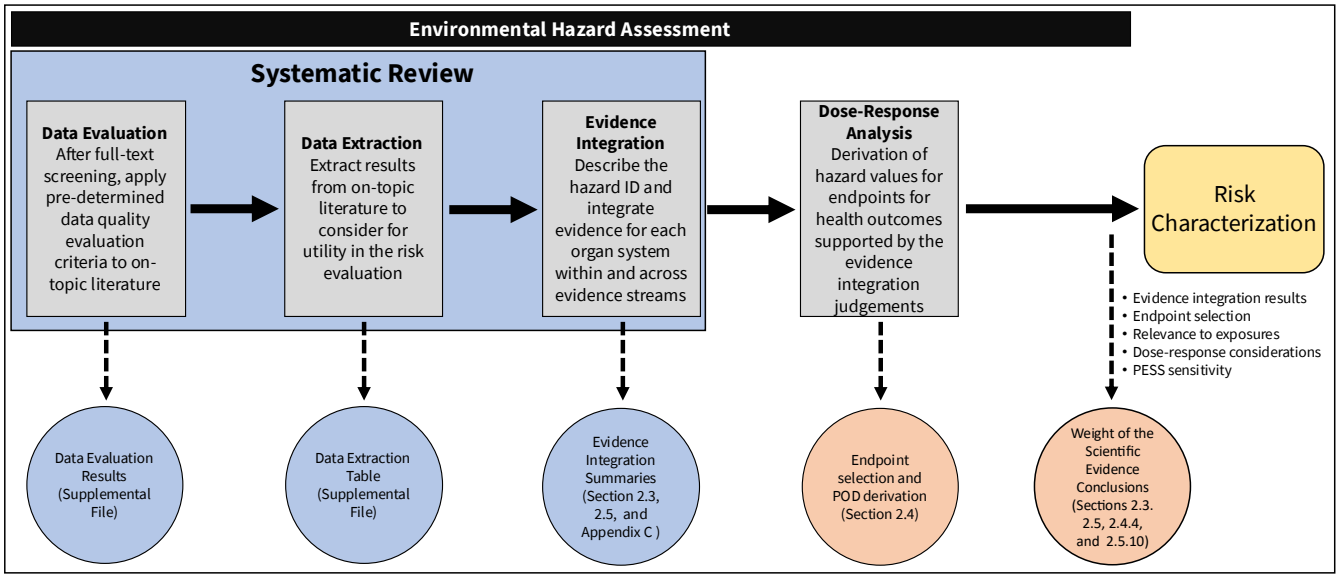

Figure 2-1. EPA Standard Approach to Hazard Identification, Evidence Integration, and Dose-Response Analysis for HHCB

2.1.1 Source Data and Evaluation

The searching and screening steps of the systematic review process for HHCB generally followed the *Draft Systematic Review Protocol Supporting TSCA Risk Evaluations for Chemical Substances, Version 1.0: A Generic TSCA Systematic Review Protocol with Chemical-Specific Methodologies* (also called the "Draft Systematic Review Protocol") (U.S. EPA, 2021) covering all reasonably available literature published through May 2025. Full details and screening results for all the identified studies are described in the *Draft Systematic Review Protocol for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (U.S. EPA, 2026j).

EPA used a refined approach to evaluate human health hazard information relevant to deriving hazard values through a filtering process to target data evaluation/extraction on key studies that may inform dose-response. All population exposure comparator and outcome (PECO)-relevant animal and epidemiological studies that passed full text screening and were not binned as "supplemental material" received data quality evaluation scores, with some exceptions as discussed below. For studies that underwent data evaluation and extraction, formal results can be found in *Draft Data Quality Evaluation Information for Human Health Hazard Animal Toxicology for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (U.S. EPA, 2026d); *Draft Data Quality Evaluation Information for Human Health Hazard Epidemiology for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-*

hexamethylcyclopenta[γ]-2-benzopyran (HHCB) (U.S. EPA, 2026e); and *Draft Data Extraction Information for Environmental Hazard and Human Health Hazard Animal Toxicology and Epidemiology for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (U.S. EPA, 2026c). For "supplemental material," basic study-level information was extracted during the filtering process and was used to support evidence integration and weight of evidence analysis.

As a fragrance chemical with direct consumer exposure, HHCB has a variety of studies not typically available in TSCA risk evaluations. EPA has appropriately made fit-for-purpose adjustments to the systematic approach for identifying and evaluating these types of studies not commonly evaluated by EPA OPPT. The Agency developed data evaluation records (DERs) for these studies as described in the *Draft Systematic Review Protocol for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (U.S. EPA, 2026j) because OPPT's data quality evaluation process is not intended to evaluate these types of *in vitro*, *in vivo*, and human studies. These studies include an OECD 443 EOGRT study in rats (IFF, 2021), a uterotrophic assay in mice (Seinen et al., 1999), three human repeated insult patch test (HRIPT) studies (IFF, 1973c, d, 1964), a dermal absorption study in humans (Ford et al., 1999), an *in vitro* dermal absorption study that was conducted in general compliance with OECD 428 (An-eX, 2001), and an OECD 439 *in vitro* dermal irritation study using reconstructed human epidermis (IFF, 2020b). The DERs for each study can be found in *Draft Data Evaluation Records for Human Health Hazard for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)*, which is a supplemental file in this draft risk evaluation (U.S. EPA, 2026b).

EPA's systematic review process additionally identified intentional dosing human studies involving dermal exposure to HHCB. These are also considered in the dermal hazard assessment (Section 2.3.2). Consistent with the approach used by EPA's Office of Pesticide Programs (OPP), when using direct exposure studies in human subjects, the Agency developed ethics reviews for these studies. These ethics reviews can be found in *Draft Ethics Reviews for Intentional Human Dosing Studies for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)*, which is also a supplemental file in this draft risk evaluation (U.S. EPA, 2026g). The human intentional dosing studies were considered for all applicable hazard outcomes depending on what was measured in the study.

The Agency performed an initial investigation of the hazard identification, critical endpoints, and key scientific issues associated with HHCB by reviewing previous assessments of the chemical. EPA's 2014 TSCA Work Plan Chemical Risk Assessment (OCSPP, 2014), a 2008 European Union risk assessment report (ECB, 2008a, b), and a tier II human health risk assessment by the National Industrial Chemicals Notification and Assessment Scheme (NICNAS) (2019) were the key federal government sources that were reviewed. Additionally, a GreenScreen® assessment prepared for Women's Voices for the Earth (ToxServices LLC, 2015) and a safety assessment from the Research Institute on Fragrance Materials (RIFM) (Api et al., 2023) served as resources for this review.

As part of the draft human health risk assessment, EPA incorporated all reasonably available information into the hazard identification, hazard characterization, evidence integration, and weight of evidence analyses. Notably, EPA identified some studies mentioned in previous assessments that were not identified by systematic review and that are not currently available to the Agency. For these studies, the Agency considered the available descriptions provided in these other assessments. EPA will update this TSD between draft and final versions with any additional information that becomes available.

2.1.2 Problem Formulation and Focus of Analysis

As discussed in the *Draft Human Exposure Assessment for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (U.S. EPA, 2026h), exposure to HHCB can occur via

inhalation, ingestion, or dermal contact. Therefore, EPA characterized the hazards of HHCB for the oral, inhalation, and dermal routes, and derived PODs for each route as appropriate to support risk estimates for the general population, consumers, and workers given the conditions of use (COUs) under TSCA identified for HHCB.

2.1.2.1 Newer Studies on Developmental and Reproductive Toxicity, Endocrine Disruption, and Dermal Irritation

As mentioned above, the Agency used a 2014 TSCA Work Plan Chemical Risk Assessment (OCSPP, 2014), a 2008 European Union risk assessment report (ECB, 2008a, b), and a tier II human health risk assessment by NICNAS (2019) as the starting points to inform this draft human health hazard assessment. Through the systematic review process, EPA did not identify any new epidemiological studies or any cancer bioassays published since these assessments. However, EPA did identify the following laboratory animal studies examining non-cancer health effects that were not discussed in previous assessments to be considered in hazard identification and/or dose-response analysis:

- an OECD 443 EOGRT (extended one-generation reproductive toxicity) study in rats (IFF, 2021);
- a reproductive/developmental toxicity screening test (modified OECD 421) that was used as a range-finding study for the above EOGRT study in rats (IFF, 2020a);
- an OECD 414 prenatal developmental toxicity study in rabbits (IFF, Date Unknown-b);
- two non-guideline studies assessing reproductive toxicity in male rats exposed via intraperitoneal (I.P.) injection (Li and Wang, 2023; Li et al., 2023); and
- an OECD 439 *in vitro* EpiSkin irritation test (IFF, 2020b).

The Agency began the assessment by focusing on the endpoints and studies considered for deriving hazard values in previous assessments. In its 2014 assessment, EPA stated that "HHCB was initially selected for review based on a moderate hazard concern for developmental toxicity and a high potential for exposure"; however, both EPA and the European Union (EU) ultimately concluded that "the overall concern for human health hazards, including that for developmental toxicity, is low" (OCSPP, 2014; ECB, 2008a, b). Both agencies identified points of departure based on developmental toxicity in rodents and acknowledged uncertainty regarding this endpoint given the lack of multigenerational reproductive toxicity studies on HHCB and given limitations in the developmental studies that were available (discussed more in Section 2.3.3 of this assessment). Therefore, EPA performed a detailed examination of the updated developmental and reproductive hazard database using the newly available EOGRT study and the other newly available studies listed above. These recent studies were considered to address uncertainties and to base the updated non-cancer hazard assessment on the best available science. The most appropriate studies and specific endpoints for hazard value derivation relevant to acute, intermediate, and/or chronic, exposure durations were then selected, and points of departure (PODs) were derived.

2.1.2.2 Mixtures Considerations

EPA's systematic review process identified several PECO-relevant dermal studies testing a common commercial mixture of HHCB in diethyl phthalate (DEP) known as Galaxolide 50. Although the 50% formulation is the industry standard for the product, many animal toxicology studies assessing Galaxolide 50 report HHCB at slightly higher concentrations (≈65%). In addition, some dermal studies involve the use of alcohol SDA 39C (Specially Denatured Alcohol; 190-proof ethyl alcohol denatured with diethyl phthalate) as a diluent. Undiluted DEP has been shown to cause slight to moderate irritation when tested on the skin of animals (Api, 2001), and alcohol SDA 39C is classified as a GHS category 2 skin irritant. EPA considered these studies as part of its dermal hazard assessment for HHCB in Section 2.3.2.

2.1.2.3 Cancer Evaluation: ReCAAP

Previous assessments did not evaluate cancer risk because no cancer bioassays were available for HHCB. EPA did not identify any new cancer bioassays published since these assessments through the systematic review process. Therefore, in Section 2.5, the Agency evaluated the weight of scientific evidence across the available studies on physical and chemical properties, toxicokinetics, acute toxicity, subchronic toxicity, genotoxicity, hormone perturbation, immune system perturbation, cancer MOA, and chronic toxicity using the Rethinking Chronic Toxicity and Carcinogenicity Assessment for Agrochemicals Project (ReCAAP Framework) (Hilton et al., 2022). By using this framework, EPA evaluated the extent to which the lack of carcinogenicity studies imparts significant uncertainty on the human health risk assessments for HHCB.

2.2 Toxicokinetics

This section describes the absorption, distribution, metabolism, and elimination (ADME) data available for HHCB. There are no HHCB toxicokinetic studies available for the oral or inhalation routes, and no physiologically based pharmacokinetic (PBPK) models were identified. Therefore, this section focuses on the available studies that characterize ADME of HHCB via the dermal route.

2.2.1 Absorption

EPA identified three studies that provide dermal absorption data for HHCB. These include an *in vitro* study in human epidermal membranes (An-eX, 2001), an *in vitro* study in a porcine back skin diffusion model (Zhang et al., 2017), and an *in vivo* study of rats and human volunteers (Ford et al., 1999). These studies are summarized in Table 2-1. In the following section, EPA evaluated these studies and made a weight of evidence-based conclusion regarding the extent to which HHCB is absorbed via the dermal route. Additionally, EPA selected the most suitable study to support its flux-based dermal exposure analysis, which can be found in Appendix C of the *Draft Human Exposure Assessment for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (U.S. EPA, 2026h).

Considerations for Interpretation of Dermal Absorption Data

Consistent with OECD's "Guidance Notes on Dermal Absorption Studies" (OECD, 2022), EPA interpreted dermal absorption data in the following manner for the available *in vitro* and *in vivo* studies. If tape strips were analyzed individually, then the amount detected in the first two tape strips is excluded from the total absorbed as it is assumed that they represent material that will not become bioavailable due to desquamation. If tape strips were pooled, then all of the material detected in the skin sample and tape strips are included in the total absorbed as a conservative estimate. If the study duration was up to 24 hours and there is evidence that the skin is not acting as a reservoir (*i.e.,* >75% permeation occurred by the midpoint of the sampling period), then all tape strips were excluded from the total absorbed.

Dermally Exposed Animals and Humans

A study by Ford et al. (Ford et al., 1999) evaluated the dermal absorption of HHCB in rats and in three human subjects. In the laboratory animal component of the study, rats (n = 18) were exposed to 0.1 mg/cm^2 HHCB dissolved in 70% ethanol for 6 hours. One hundred percent of the applied radioactivity was recovered. Most of the applied dose was detected at the site of application in dressings and skin washings (79.3%) after 6 hours and in the post-exposure dressing (5.7%) at 120 hours. At 120 hours, the mean dermal absorption of radiolabeled HHCB was reported to be 13.7% of the applied dose and was calculated based on the percent detected in tissues (0.7%) and excreta (13%). Notably, though 2.02% of the applied dose was detected in the skin, the study authors did not include this amount in the total absorbed dose.

According to OECD's "Guidance Notes on Dermal Absorption Studies" (OECD, 2022), " If during an *in vivo* animal study there is measurable ongoing depletion of the dose from the application site following washing and a corresponding increase in cumulative absorbed dose over time, the dose remaining at the application site, including all material in the stratum corneum (perhaps excluding the upper two tape strips), is considered to be available for further skin absorption." During the study by Ford and colleagues, the amount of radioactivity in the skin decreased steadily from 10% at 6 hours to 2% at 120 hours, and the total radioactivity in the tissues and excreta increased from 3.89% at 6 hours to 13.7% at 120 hours. Therefore, EPA determined that the percentage of applied HHCB that was absorbable, including the amount in skin, was 15.7% (13.7 + 2.02% detected in skin). Given that rodent skin is more permeable than human skin, this study likely over-estimates the percentage of HHCB that was absorbed dermally. Because of this uncertainty and the availability of information on human dermal absorption, this study was deemed inappropriate for deriving a dermal absorption factor or for calculating dermal flux.

In the same publication, three human volunteers were exposed to an average dose of 0.018 mg/cm^2 body area dissolved in 70% ethanol for 6 hours. Similar to the rat study, most of the radiolabeled HHCB was recovered at the site of application in dressing and skin washes (55.69%) at 6 hours and in the post-exposure dressing at 120 hours (19.54%). At 120 hours, the mean dermal absorption of radiolabeled HHCB was reported to be less than 0.1% and was calculated based on the percent of applied HHCB detected in urine (0.1% in 1 sample from 1 individual) and feces (below the limit of detection at all sampling times in all individuals) at 120 hours. Skin was tape-stripped five times immediately after dose removal at 6 hours and then again at 120 hours. Notably, 10.97% of the applied dose was detected in the pooled 6-hour tape strips and 0.27% was detected in the pooled 120-hour tape strips; it is uncertain whether these amounts in the skin could be absorbed over time. Additionally, recovery ranged from 71 to 78% of the applied radioactivity across the three human subjects; therefore, it is uncertain whether any of the remaining 22 to 29% was absorbed and deposited in the tissues and/or remained in deeper layers of the skin. However, this is unlikely given that 22% of applied HHCB evaporated in a separate experiment testing evaporation in the same study. Additionally, a study in human epidermal membranes (discussed in the section below) similarly found that only 0.6% of applied HHCB permeated past the skin and detected only 5% to 8% within skin depending on whether radioactivity in tape strips was included (An-eX, 2001). Nevertheless, given that this study in human subjects was not designed to account for amounts of HHCB retained in deeper layers of the skin, which could be absorbable over time or any amounts in tissues, this study likely somewhat underestimates the percentage of HHCB that is absorbed dermally and was deemed inappropriate for deriving a dermal absorption factor or for calculating dermal flux.

In Vitro Studies in Epidermal Membranes

In two *in vitro* studies, absorption of HHCB ranged from 5.2 to 11.4% depending on the study and skin partitions factored into the absorbed dose. Both studies are discussed below.

A study using a porcine back skin diffusion model reported that 11.4% of the administered dose of HHCB was absorbed at 24 hours based on the amount detected in the skin layers below the stratum corneum (11.1%) and in the receptor fluid (0.3%) (Zhang et al., 2017). The authors stated that total recovery was greater than 85%, though this total is uncertain given that the breakdown of contributions to this total were not shown (combined amount in all skin layers plus receptor fluid appears to be between 30 and 40% from graphs; the amounts of evaporated material captured by sponges and amounts of material in skin washes were not presented). This study failed to report several additional details that add uncertainty regarding the results. Namely, all results were only graphically presented, it is unclear whether integrity of the skin samples was assessed, it is unclear whether HHCB was administered neat

or dissolved in a vehicle, and the experimental set up of the Franz diffusion cell was not described. Furthermore, the authors did not use an appropriate receptor fluid (authors used water as receptor fluid even though HHCB is lipophilic [log K_{OW} = 5.9]). Because of these uncertainties and limitations, this study was deemed inappropriate for deriving a dermal absorption factor or for calculating dermal flux.

A study in human epidermal membranes that pre-dated OECD 428 but was similar in design reported that 5.2% of the administered dose of HHCB was absorbed at 24 hours based on the amount of radioactivity detected in the skin excluding all tape strips (4.52%), in receptor fluid (0.397%), as well as in filter paper under the skin samples (0.245%) (An-eX, 2001). Similar to the rat and human subject studies described above, most of the radiolabeled HHCB was recovered in the surface wipe (58.2%) and donor chamber after washing (22.9%). Total recovery was 92% and was closer to 100% when factoring the percentage that evaporated (2.4%) in a separate experiment, which assessed the evaporative loss from polytetrafluoroethylene (PTFE) sheets. However, it is important to note that the study authors did not factor the amounts of HHCB detected in any tape strips into the absorbed dose, which is recommended by OECD's "Guidance Notes on Dermal Absorption Studies" (OECD, 2022) when dermal absorption is not complete. Specifically, the OECD guidelines state that "For *in vitro* studies, permeation is considered essentially complete when more than 75% of the amount that has permeated into the receptor fluid at the end of sampling (usually at 24 hours) has reached the receptor fluid at the half time of the sampling period (usually at 12h)." This was not the case in this study: At 12 hours, a mean of 0.242 µg/cm^2 was detected in the receptor fluid, and at 24 hours, a mean of 0.795 µg/cm^2 was detected. Therefore, percentages of radioactive HHCB detected in tape strips 2 through 10 were added to the absorbed dose reported by the study authors. Taking these factors into consideration, EPA determined that the total absorbable dose of HHCB is 8.85%.

639 **Table 2-1. Summary of HHCB Studies Evaluating Dermal Absorption**

Reference	Brief Study Description	Percent Absorbed at Study Conclusion [a]	Study Quality Rating
(Ford et al., 1999)	Male Lister-Hooded (pigmented) rats (n = 18) exposed to radiolabeled HHCB dissolved in 70% ethanol at a nominal dose of 4 mg/kg (0.1 mg/cm^2 over 9 cm^2 in a volume of 200 µL) for 6 hours under occluded conditions. At 6 hours (or at sacrifice if before then), test substance was removed using swabs moistened with 70% ethanol. Skin was then re-occluded with a fresh dressing. Pairs of animals were sacrificed at 0.5, 1, 3, 6, 12, 24, 48, 72 and 120 hours after dose administration. HHCB content was measured via liquid scintillation counting (LSC) in urine, feces, blood, tissues, skin, skin washes, dressings, and cages.	15.7% (includes amounts in skin, tissues, and excreta)	Uninformative
	Three male human volunteers exposed to radiolabeled HHCB dissolved in 70% ethanol at a nominal dose of 4.0 mg/mL for 6 hours without occlusion. After the 6-hour exposure period, gauze was removed, and test material was washed from the surface of the skin with cotton wool swabs moistened with 70% ethanol. Skin was tape stripped, covered with fresh gauze, and then tape stripped again at 120 hours. HHCB content was measured via LSC in blood, feces and urine were collected over a 5-day period, as well as in tape strips, gauze dressings, and swabs.	11.28% (includes amounts in 6- and 120-hour tape strips and excreta)	Acceptable/non-guideline [b]
(Zhang et al., 2017)	100 ng HHCB was applied to porcine back skin samples (n = 4–5 replicates per time point, each with an area of 2.26 cm^2) for 4, 6, 12, 18, or 24 hours. After each of these time points, skin was washed with water and tape stripped. HHCB content was measured via GC/MS (gas chromatography-mass spectrometry) in sponges used to capture volatilized test substance, tape strips, remaining skin, skin washes, and receptor fluid.	11.4% (includes amounts in tape strips, remaining skin, and receptor fluid)	Uninformative
(An-eX, 2001)	Radiolabeled HHCB was applied as a 20µL/cm^2 target dose of a 1% solution in ethanol to human epidermal membranes (n = 12 replicates) under non-occlusive conditions for 24 hours. HHCB content was measured in the receptor phase at 1, 2, 6, 12, and 24 hours via LSC. After the 24-hour timepoint, residual chemical was removed with a dry swab and membranes were tape stripped. Radiolabel content of the swabs, tape strips, remaining skin, filter paper below the skin, and washed diffusion cells were determined. Evaporative loss of HHCB was estimated by measuring the loss from polytetrafluoroethylene (PTFE) sheets under the same experimental conditions. OECD TG 428	8.85 % (includes amounts in tape strips, remaining skin, filter paper, and receptor fluid)	Acceptable/non-guideline [b]

[a] Absorbed percentages reported in this table are reflective of EPA's determination of the absorbable dose using OECD guidance on dermal absorption studies (OECD, 2022) and do not always match the absorbed dose reported by the study authors. In cases where these percentages differ, both values are provided in the text of this section along with a discussion of uncertainties associated with each value.
[b] Reference was evaluated using the OPP DER format.

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Conclusions on Dermal Absorption

The available studies in humans and animals indicate that HHCB has low dermal absorption, with a lower bound of less than 0.1% based on a study in human subjects, and an upper bound of 5.2 to 8.1% based on a study in human skin samples. Furthermore, upon evaluating the strengths, limitations, and uncertainties of the available dermal absorption studies, EPA selected the *in vitro* human skin absorption study (An-eX, 2001) to support dermal flux calculations in the *Draft Human Exposure Assessment for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (U.S. EPA, 2026h). This is because the study was conducted in human skin samples, was generally consistent with OECD 428, and included time-course data to support calculation of dermal flux. The rationale for EPA's flux-based approach and the calculation of dermal flux are described in more detail in Appendix C of the *Draft Human Exposure Assessment for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (U.S. EPA, 2026h).

2.2.2 Distribution

Data from Dermally Exposed Animals

In the rat *in vivo* dermal absorption study described above (Ford et al., 1999), distribution of radioactivity was measured during 0.5 to 120 hours after dermal application of ^{14}C-labeled HHCB. The highest levels of radiolabeled HHCB were detected in the intestines and their contents, which peaked at 4% of the applied dose at 24 hours. By 120 hours, 0.7% of the applied dose was present in tissues (specifically in fat, liver, intestine contents, and other tissues).

Data from Animals Exposed via Intravenous (IV) Injection

In a study conducted by Api et al. (2013), rats received a single intravenous dose of 2 mg/kg body weight of radiolabeled HHCB in the tail vein and distribution of radioactivity was measured at timepoints ranging from 5 minutes to 28 days in the liver, kidney, and fat. The highest level of radiolabeled HHCB was detected in the liver, which peaked at 8.83 ug/g tissue at 5 minutes. Lower levels of radiolabeled HHCB were detected in the kidney and fat (4.65 ug/g tissue at 5 minutes and 6.62 ug /g tissue at 2 hours, respectively). By 7 days post-exposure, levels decreased to 0.1, 0.02, and 0.58 ug/g tissue in the liver, kidney, and fat, respectively.

In this same study (Api et al., 2013), a domestic pig (*Sus scrofa*; n = 1) received a single intravenous dose of 0.1 mg/kg body weight of radiolabeled HHCB and radioactivity was measured at timepoints ranging from 9 days to 28 days in the fat and skin. In fat, the maximal concentration was at 9 days. After that, the fat concentration decreased slowly and was less than 3.1 ng/g tissue 16 days after injection and less than 0.5 ng/g tissue after 28 days. In skin, the maximal concentration was at 9 days (3.8 ng/g) declining to 0.8 ng/g at 16 days and to below the limit of accurate measurement (<0.5 ng/g) at 28 days.

Biomonitoring Data in Humans

HHCB has been detected in human cord blood, breastmilk, and adipose tissue in limited numbers of the general populations in the U.S., Asia, and Europe. Details on these studies can be found in EPA's 2014 TSCA Work Plan Chemical Risk Assessment of HHCB (OCSPP, 2014) and in the *Draft Human Exposure Assessment for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (U.S. EPA, 2026h). Specifically, one U.S. study detected HHCB in adipose tissue at mean levels of 178 ng/g lipid weight (lw) (range: 12–798 ng/g lw) in 49 residents of New York City (Kannan et al., 2005). Mean levels in adipose tissue from studies outside of the United States ranged from 81 ng/g lipid weight to 361 ng/g lipid weight. Mean levels detected in breast milk ranged from 0.055 to 227 ng/g lipid weight across studies from various countries, with the latter amounts measured in 39 milk samples collected in Massachusetts (range: <5 to 917 ng/g lw) (Reiner et al., 2007). No data on blood concentrations, including umbilical cord blood, are available in U.S. populations; studies reporting

concentrations of HHCB in blood are so variable that EPA (OCSPP, 2014) recommended that they cannot and should not be compared.

These results are limited by high variability in measurements within studies (likely due to small sample sizes and ongoing developments in analytical methods for measuring HHCB in human matrices) and across studies (likely due to different patterns of use across different countries). Still, HHCB was detected in a majority of the samples collected in the studies reported here. Therefore, while this data cannot be extrapolated to the general U.S. population, it supports data from animal toxicokinetic studies demonstrating that HHCB distributes to fatty tissues. It also confirms that HHCB has the potential to be transferred to the developing fetus via cord blood and to nursing children.

2.2.3 Metabolism

Available data regarding metabolism of HHCB comes from one study that exposed rats and a single pig to a single dose via intravenous injection (Api et al., 2013). In both species, the HHCB that was detected in urine was completely metabolized. The feces were not analyzed for parent compound vs. metabolites.

Although the metabolites were not fully characterized, thin-layer chromatography (TLC) profiles revealed that, while there were quantitative differences in the concentration of metabolites between rat and pig, metabolites present in rat urine and pig urine had similar retention times suggesting that the pig and the rat produce primarily the same metabolites. Nine discrete radioactive components were detected in rat urine, and enzyme treatment (β-glucuronidase) revealed two additional components. Twelve radioactive components were identified in pig urine regardless of enzyme treatment. In the absence of enzyme treatment, the major metabolite was the same in rat and pig urine, whereas after enzyme treatment, the major metabolite differed between the two species.

2.2.4 Elimination

Dermally Exposed Animals and Humans

In the rat *in vivo* dermal absorption study described above (Ford et al., 1999), 11.6 and 1.24% of administered radiolabeled HHCB was recovered in the feces and urine, respectively, at 120 hours. No significant excretion through expired air was detected. In the same study, amounts of HHCB recovered in urine and feces of three human volunteers was below the limit of detection (0.008–0.23% and 0.023–0.15% of applied radioactivity for urine and feces, respectively) in all samples except for a 12- to 24-hour urine sample in one subject.

Animals Exposed via Intravenous Injection

A study that measured urinary and fecal excretion of HHCB in rats and in a pig after intravenous injection suggests that HHCB is primarily excreted in the urine of rats and the feces of pigs (Api et al., 2013). Specifically, in rats that received a single intravenous dose of 2 mg/kg body weight of radiolabeled HHCB in the tail vein, 61 and 28.1% of the radiolabeled material was detected in the feces and urine, respectively, over the entire collection period (168 hours). In the same study, 74 and 14.6% was excreted in the urine and feces, respectively, over the entire collection period of 336 hours in a pig that received a single intravenous dose of 0.1 mg/kg body weight of radiolabeled HHCB. Respiratory excretion was not measured for either species.

2.3 Non-Cancer Hazard Assessment

This section summarizes the non-cancer hazards associated with inhalation (Section 2.3.1), dermal (Section 2.3.2), and oral (Section 2.3.3) exposure to HHCB. This section focuses on the primary human health hazards for each route of exposure. Additional hazard outcomes are discussed in Appendix C.

2.3.1 Inhalation Route

2.3.1.1 Acute Inhalation Toxicity

No data are available for the inhalation route that are suitable for deriving route-specific PODs. EPA identified one acute inhalation (OECD 403) toxicity study in rats for HHCB (IFF, 2017). In this study, Wistar rats (n = 5 females and 5 males) were exposed to a single concentration of HHCB in an aerosol atmosphere (50:50 w/w HHCB: ethanol) for 4 hours using a nose only exposure system, followed by a 14-day observation period. The mean achieved atmosphere concentration of HHCB was 5.04 mg/L. No deaths occurred. Clinical observations indicated that decreased respiratory rate, ataxia, hunched posture, pilo-erection, and wet fur were observed; however, animals appeared normal on day 2 post-exposure. Transient body weight losses or no gain in body weight were noted on day 1 post-exposure in four males and four females, on days 1 to 3 in two females, and from days 3 to 7 in one female; however, body weight gains were noted throughout the remainder of the recovery period for all animals. No macroscopic abnormalities were detected at necropsy. This study concluded that the 4-hour LC50 (lethal concentration at which 50% of test organisms die) exceeded 5.04 mg/L. No additional laboratory animal or epidemiological studies were available for the inhalation route.

2.3.2 Dermal Route

In the following sections, EPA assessed human, laboratory animal, and mechanistic data and integrated these evidence streams for the following dermal hazard outcomes: acute dermal lethality, subchronic dermal toxicity, dermal irritation, and dermal sensitization. The Agency then integrated conclusions across these hazard outcomes to make an overall conclusion regarding whether HHCB is hazardous via the dermal route.

Multiple studies via the dermal route are available. Some involve neat HHCB where others include HHCB combined with other compounds. Some of these studies involve Galaxolide 50, a common mixture found with DEP. As a solvent, carrier, and fixative in fragrance products, the presence of DEP enhances the skin penetration of lipophilic compounds like HHCB. Undiluted DEP has been shown to cause slight to moderate irritation when tested on the skin of animals (Api, 2001). In addition, some studies involve the use of alcohol SDA 39C as a diluent. Alcohol SDA 39C is classified as a GHS category 2 skin irritant. Therefore, for each dermal hazard outcome, EPA first considered evidence from studies of neat HHCB. EPA then considered studies involving mixtures with DEP or alcohol SDA 39C.

EPA identified the following studies assessing neat HHCB or HHCB dissolved in petroleum jelly:

- an OECD 402 acute toxicity test in rats (IFF, 2016a);
- one human repeated insult patch test (HRIPT) (IFF, 1973c);
- an OECD 439 *in vitro* EpiSkin irritation test (IFF, 2020b); and
- a patch test in fragrance-sensitive human subjects (Larsen et al., 2001).

EPA identified the following studies assessing Galaxolide 50. Notably, some of these studies are not currently available to the Agency and were instead identified in previous assessments (NICNAS, 2019; OCSPP, 2014; ECB, 2008a, b). For studies cited by previous assessments that are currently unavailable, EPA considered the conclusions provided in these other assessments in evaluating the weight of evidence. The Agency will update this evaluation between draft and final with any additional information that becomes available.

- A pre-guideline acute dermal toxicity test in Sprague-Dawley (SD) rats exposed to Galaxolide 50 (65% HHCB in DEP) described in previous assessments.

- A pre-guideline acute dermal toxicity test in albino rabbits exposed to Galaxolide 50 (65% HHCB in DEP) described in previous assessments.
- A non-guideline subacute dermal toxicity study in SD rats exposed to Galaxolide 50 (65% HHCB in DEP) further diluted in ethanol of for 26 weeks (IFF, Date Unknown-c). Concentrations of HHCB were 0, 32.5, 65, and 130 mg/kg-day.
- A non-guideline subacute dermal toxicity study in SD rats exposed to Galaxolide 50 (65% HHCB in DEP) further diluted in ethanol for 13 weeks described in previous assessments. Concentrations of HHCB were 0.65, 6.5, and 65 mg/kg-day.
- A non-guideline subacute dermal toxicity study in SD rats exposed to Galaxolide 50 (65% HHCB in DEP) further diluted in ethanol for 26 weeks described in previous assessments. Concentrations of HHCB were 5.85, 11.7, and 23.4 mg/kg-day.
- A patch test in fragrance-sensitive human subjects exposed to Galaxolide 50 (percentage of HHCB and solvent not specified) further diluted in petroleum jelly (An et al., 2005).

EPA identified the following studies assessing HHCB diluted in alcohol SDA 39C: Two human repeated insult patch tests (HRIPT) (IFF, 1973d, 1964)

2.3.2.1 Acute Dermal Toxicity

2.3.2.1.1 Laboratory Animal Studies Testing Neat HHCB

Evidence regarding the systemic dermal toxicity of HHCB after acute exposure comes from one laboratory animal study. In an acute dermal toxicity test that was conducted in general compliance with OECD 402, SD rats (n = 5 per sex per treatment group) were exposed to 2,000 mg/kg undiluted Galaxolide (100% HHCB) or water on intact shaved skin for 24 hours using non-occlusive gauze dressing (IFF, 2016a). After the 24-hour exposure period, gauze dressings were removed, and the area was rinsed with distilled water. Animals were then observed for 14 days. Slight erythema (*i.e.,* a score of 1) was reported in all females between days 2 and 5; no edema was reported. The following outcomes were unaffected by HHCB up to 14 days post-treatment: mortality, systemic clinical signs, body weight, and macroscopic examinations of organs. Therefore, a dermal LD50 exceeding 2,000 mg/kg HHCB was reported.

2.3.2.1.2 Laboratory Animal Studies Testing Galaxolide 50 (HHCB in DEP)

Two additional studies that are not available to EPA assessed the systemic toxicity of Galaxolide 50 (a mixture of HHCB in DEP) in rats and rabbits, respectively, after acute dermal exposures. These were summarized by previous assessments, though many details were unavailable (NICNAS, 2019; OCSPP, 2014; ECB, 2008a, b).

In the first study, Galaxolide 50 (65% HHCB in DEP) was administered undiluted by inunction to the shaved skin (area not reported) of groups of five female SD rats. Final doses of HHCB were reported to be 300, 650, 1,400, 3,000, and 6,500 mg/kg. Animals were observed for 7 days. There were no deaths at any dose, but all animals in the high dose group exhibited urine staining on their fur. A dermal LD50 (lethal dose) exceeding 6,500 mg/kg bw HHCB was reported. This study was conducted prior to OECD guidelines but was conducted according to acceptable procedures at the time.

In the second study, Galaxolide 50 (65% HHCB in DEP) was applied to the skin of groups of seven albino rabbits. The final dose of HHCB was reported to be 3,250 mg/kg. There were no deaths at that dose; therefore, a dermal LD50 exceeding 3,250 mg/kg bw HHCB was reported. This study was conducted prior to OECD guidelines and specific details regarding the study are not available. However,

822 previous assessments report that the study was conducted in general compliance with OECD Guideline
823 401 according to a personal communication with RIFM (ECB, 2008a, b).

824 **Table 2-2. Summary of HHCB Studies Evaluating Acute Dermal Toxicity in Animals**

Reference(s)	Study Description	LD_{50} (mg/kg)	Effects	Study Quality Rating
(IFF, 2016a)	SD rats (n = 5 per sex per treatment group) exposed to 2,000 mg/kg undiluted Galaxolide (100% HHCB) or water on intact skin for 24 hours. OECD 402	>2,000	None except for slight erythema (*i.e.,* score of 1) in all females between days 2 and 5	High
(NICNAS, 2019; OCSPP, 2014; ECB, 2008a, b)[a]	SD rats (n = 5 females per treatment group) exposed to Galaxolide 50 (65% HHCB in DEP) at final HHCB doses of 300, 650, 1400, 3000, and 6500 mg/kg	>6,500	None	N/A
(NICNAS, 2019; OCSPP, 2014; ECB, 2008a, b) [a]	Albino rabbits (n = 7 per treatment group) exposed to Galaxolide 50 (65% HHCB in DEP) at a final HHCB dose of 3,250 mg/kg	>3,250	None	N/A

DEP = diethyl phthalate; LD_{50} = lethal dose at which 50% of test organisms die; SD = Sprague-Dawley (rats)
[a] Study not available to EPA but is summarized in previous assessments.

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Evidence Integration Summary

Strong evidence supports no systemic effects from acute dermal exposure to HHCB. This conclusion is supported by robust evidence from animal studies. Specifically, the available OECD 402 acute dermal toxicity study in rats did not identify any adverse effects of exposure to neat HHCB at doses as high as 2,000 mg/kg. Additionally, two pre-guideline studies testing mixtures of HHCB in DEP determined that LD50s for HHCB were greater than 6,500 and 3,250 mg/kg.

This conclusion is consistent with other regulatory agencies. Specifically, U.S. EPA (OCSPP, 2014) concluded that "the acute toxicity of HHCB is low via the dermal route in rabbits, with LD50 values exceeding 3,000 mg/kg body weight"; NICNAS (2019) concluded that "the chemical is expected to have low acute toxicity via the dermal route" ; and EU (ECB, 2008a, b) concluded that "There is no need to classify HHCB for acute toxicity" for all exposure routes, including dermal."

2.3.2.2 Subchronic Dermal Toxicity

Evidence from Laboratory Animal Studies Testing Galaxolide 50 (HHCB in DEP)

In a non-guideline subacute dermal toxicity study, Galaxolide 50 diluted in ethanol was applied daily to the shaved backs of SD rats (n = 15 males and 35-38 females per dose) at concentrations of 0, 50, 100, or 200 mg/kg-day of for 26 weeks (IFF, Date Unknown-c). Given that Galaxolide 50 is a mixture of 65% HHCB, the equivalent concentrations of HHCB were 0, 32.5, 65, and 130 mg/kg-day. A vehicle control group received ethanol at a volume equal to the largest volume administered to a test group. At the highest doses (65 and 130 mg/kg-day) some rats displayed scabbed areas and appearance of a white or brown crusty material. No statistically significant effects were observed on body weights, hematology, biochemical parameters or urinalysis. Organs were unaffected except for an increase in relative liver weight in females at week 26 (11 and 23% in the 65 and 130 mg/kg-day dose groups, respectively) and kidney weights (37% in males receiving the highest dose). No histopathological defects were noted in liver or kidney. Notably, this study was not designed to distinguish effects of HHCB from those of DEP and lacked a description of measures taken to prevent ingestion of the test material.

Two additional studies that are not available to EPA assessed the systemic toxicity of Galaxolide 50 in rats after subchronic dermal exposures. These were summarized by previous assessments, although many details were unavailable (NICNAS, 2019; OCSPP, 2014; ECB, 2008a, b). In the first study, female SD rats (n = 15 per dose) received topical applications of Galaxolide 50 at 1, 10 or 100 mg/kg-day as a 2% solution in ethanol for 13 weeks. Given that Galaxolide 50 is a mixture of 65% HHCB, the equivalent concentrations of HHCB were 0.65, 6.5, and 65 mg/kg-day. There were no reported adverse clinical signs, no variation in biochemistry or hematological parameters, no effects on body weight, and no histological changes at any dose. Increases in absolute and relative liver weights in the highest dose group were reported; however, details of the magnitude of these changes are not available. In the second study, female SD rats (n = 20 per dose) received topical applications of Galaxolide 50 at 9, 18, or 36 mg/kg-day as a 2% solution in ethanol for 26 weeks. Equivalent concentrations of HHCB were 5.85, 11.7, and 23.4 mg/kg-day. There were no reported adverse clinical signs, no variation in biochemistry or hematological parameters, and no histological changes at any dose. Body weight decreases were reported in the highest dose group; however, the magnitude of these effects was not reported, and their significance cannot be determined. No measures were taken to prevent oral ingestion of the chemical in these studies.

871 **Table 2-3. Summary of HHCB Studies Evaluating Dermal Toxicity After Repeated Subchronic Exposure in Animals**

Reference	Study Description	NOAEL/LOAEL (mg/kg-day)	Effects	Study Quality Rating
(IFF, Date Unknown-c)	SD rats (n = 15 males and 35-38 females per dose) exposed to 0, 50, 100, or 200 mg/kg-day of Galaxolide 50 (65% HHCB in DEP) diluted in ethanol daily for 26 weeks. This equates to 32.5, 65, and 130 mg/kg-day HHCB.	130/ND	Increased relative liver weight in females but not males that was not considered adverse (See Appendix C.2)	High
(NICNAS, 2019; OCSPP, 2014; ECB, 2008a, b) [a]	Female SD rats (n = 15 per dose) received topical applications of Galaxolide 50 (65% HHCB in DEP) at 1, 10 or 100 mg/kg-day as a 2% solution in ethanol for 13 weeks. This equates to 0.65, 6.5, and 65 mg/kg-day HHCB.	65/ND	None	N/A
(NICNAS, 2019; OCSPP, 2014; ECB, 2008a, b) [a]	Female SD rats (n = 20 per dose) received topical applications of Galaxolide 50 (65% HHCB in DEP) at 0, 9, 18, or 36 mg/kg-day as a 2 % solution in ethanol for 26 weeks. This equates to 5.85, 11.7, and 23.4 mg/kg-day HHCB.	23.4/ND	None	N/A

DEP = diethyl phthalate; ND = dot determined; SD = Sprague-Dawley (rats)
[a] Study not available to EPA but is summarized in previous assessments.

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Evidence Integration Summary

Strong evidence supports no systemic effects from subchronic dermal exposure to HHCB. This conclusion is supported by robust evidence from animal studies testing mixtures of HHCB in DEP. Specifically, the available studies assessing dermal toxicity of Galaxolide 50 after subchronic exposure did not identify any adverse effects in rats at doses of HHCB as high as 130 mg/kg-day.

This conclusion is consistent with other regulatory agencies. Specifically, regarding repeated dose toxicity of HHCB, EU (ECB, 2008a, b) concluded that "given the lack of significance in the findings reported in two studies and the lack of an adverse effect dose in the third, it is impossible to conclude a true NOAEL in terms of dermal toxicity." NICNAS (2019) concluded that "based on the available information, the chemical is not expected to cause serious damage to health from repeated dermal exposure."

2.3.2.3 Dermal Irritation

2.3.2.3.1 Human Evidence from Neat HHCB

Available studies evaluating the skin irritation potential of neat HHCB in humans includes one HRIPT (IFF, 1973c), as well as an OECD 439 *in vitro* EpiSkin irritation test (IFF, 2020b).

In the induction phase of an HRIPT study (IFF, 1973c), 42 subjects (40 female and 2 male) were patch-tested with 100% neat HHCB under semi-occlusive dressing. Patches were applied to the upper arms for 24 hours for a total of nine induction applications. The schedule of when patches were applied and scored for each subject was redacted; however, a similar study by the same group specifies that induction patches were applied three times per week for 3 weeks (IFF, 1964). Subjects were instructed to remove the patches 24 hours after application, and skin was evaluated according to the Draize method on the day of the next scheduled application. No irritation was observed in any subjects treated with 100% HHCB.

In an OECD 439 *in vitro* study using a reconstructed human epidermis model (IFF, 2020b) triplicate tissues per treatment group were treated with "HHCB/undiluted Galaxolide," Dulbecco's phosphate buffered saline (negative control), or Sodium dodecyl sulphate (positive control) for 15 minutes without occlusion. Notably the test substance was reported to be 70% pure, and the other constituents identified in the certificate of analysis were not named. Cytotoxicity was measured via the colorimetric MTT reduction assay 48 hours later. The relative mean viability of the treated tissues was 76.9%, and the authors classified the test item as non-irritant.

Table 2-4. Summary of HHCB Studies Evaluating Effects on Dermal Irritation in Humans

Reference	Study Description	Effects	Study Quality Rating[a]
(IFF, 1973c)	42 subjects (40 female and 2 male) were tested for irritation as part of a sensitization study. During induction, subjects were patch- tested with neat Galaxolide (100% HHCB). Patches applied to the upper arms for 24 hours 3 times per week for 3 weeks. Skin was evaluated according to the Draize method.	No irritation observed in all subjects	Acceptable/Non-guideline
(IFF, 1973d)	43 subjects (36 female and 7 male) were tested for irritation as part of a sensitization study. During induction, subjects were patch- tested with 50% HHCB diluted in alcohol SDA 39C and with vehicle control. Patches applied to the upper arms for 24 hours 3 times per week for 3 weeks. Skin was evaluated according to the Draize method.	Reversible Grade 1 reaction (slight erythema) in one HHCB-treated subject and in one subject treated with alcohol SDA 39C vehicle during induction exposures	Acceptable/Non-guideline
(IFF, 1964)	40 subjects (28 female and 12 male) were tested for irritation as part of a sensitization study. During induction subjects were patch-tested with 3.75% HHCB diluted in alcohol SDA 39C. Patches applied to the upper arms for 24 hours 3 times per week for 3 weeks. Skin was evaluated according to the Draize method.	Grade 1 and 2 reactions in 7 (17.5%) HHCB-treated subjects that also occurred in a separate study in 13-15% of the vehicle-treated population when semi-closed patch testing was used and in 33% when occlusive patch testing was used for the first three induction applications	Acceptable/Non-guideline
(IFF, 2020b)	EpiSkin reconstructed human epidermis model (n = 3 triplicate tissues per treatment group) treated with undiluted Galaxolide (purity was reported to be 70%) for 15 minutes. Cytotoxicity was measured via the colorimetric MTT reduction assay 48 hours later. OECD 439	The relative mean viability of the treated tissues was 76.9% and the authors classified the test item as non-irritant.	Acceptable/Guideline

SDA = Specially Denatured Alcohol (39C)
[a] References were evaluated using the OPP DER format.

2.3.2.3.2 Laboratory Animal Evidence from Neat HHCB

One study evaluated the skin irritation potential of neat HHCB in laboratory animals (IFF, 2016a). In an acute dermal toxicity test, SD rats (n = 5 per sex per treatment group) were exposed to 2,000 mg/kg undiluted Galaxolide (100% HHCB) or water on intact skin for 24 hours under non-occlusive dressing. "Slight erythema between days 2 and 5" was observed in all females, but no males, relative to control animals. No edema was observed in either sex relative to control. Notably, although the authors stated that this study was conducted in general compliance with OECD guideline 402, this study had limitations that complicated the interpretation of the observed effects. Specifically, the scoring methods and the individual reaction scores for each animal on each day were not provided; therefore, it is impossible to determine from the existing description of the results whether any erythema reactions noted during the observation period also recovered during that period (days 2 to 5). Additionally, the study guideline recommends that measurements should be taken up to 14 days post-treatment to determine reversibility of effects. Finally, data from the control animals that were exposed to water were not provided, making it impossible to compare the results in HHCB-exposed rats to incidental levels of slight erythema.

2.3.2.3.3 Human Evidence from HHCB Diluted in Alcohol SDA 39C

The dermal irritation potential of HHCB was tested during the induction phase of two additional HRIPT studies testing HHCB diluted in alcohol SDA 39C (1973d, 1964). These studies are discussed below.

In a study by IFF (1973d), 43 subjects (36 female and 7 male) were patch-tested with 50% HHCB diluted in alcohol SDA 39C and with vehicle control under semi-occlusive dressing. Patches were applied to the upper arms for 24 hours for a total of nine induction applications. The schedule of when patches were applied and scored for each subject was redacted from the study; however, the second study described below, which involved a similar design, specifies that induction patches were applied three times per week for 3 weeks. Subjects were instructed to remove the patches 24 hours after application, and skin was evaluated according to the Draize method on the day of the next scheduled application. A reversible Grade 1 reaction (slight erythema) was observed in one subject treated with 50% HHCB, as well as in a different subject treated with alcohol SDA 39C vehicle in the same experiment.

In a second study by IFF (1964) 40 subjects (28 female and 12 male) were patch-tested with 3.75% HHCB diluted in alcohol SDA 39C under semi-occlusive dressing, with the exception of 4 subjects that received occlusive dressings for the first three applications. Patches were applied to the upper arms for 24 hours for a total of nine induction applications. Induction patches were applied three times per week for 3 weeks. Subjects were instructed to remove the patches 24 hours after application, and skin was evaluated according to the Draize method on the day of the next scheduled application.7 subjects (17.5%) had reaction sites scored with a Grade of 1 or 2 throughout the induction phase. No reaction sites scored higher than Grade 2 at any time. A separate study examining the vehicle control and/or the use of occlusive patch testing that is attached to end of the report indicates a potential confounding effect of alcohol SDA 39C. Specifically, Grade 1 to 2 reactions occurred in 13 to 15% of the vehicle-treated population when semi-closed patch testing was used and in 33% when occlusive patch testing was used for the first three induction applications. Therefore, reactions observed in the seven 3.75% HHCB-treated participants were potentially related to the vehicle and/or use of occlusive patches rather than to HHB treatment.

2.3.2.3.4 Laboratory Animal Evidence from Galaxolide 50 (HHCB in DEP)

Two acute dermal irritation/corrosion studies in rabbits evaluated mixtures of HHCB diluted in DEP (known as Galaxolide 50) (IFF, 1975a, 1973b). Specifically, in the first study conducted by IFF (1973b) albino rabbits (sex and strain/stock not specified; n = 3 per treatment group) were exposed to Galaxolide 50 (final concentration of 70% HHCB in DEP) on intact and abraded skin for 24 hours under occlusive dressing. Immediately after 24 hours and again 48 hours later, skin was evaluated according to the Draize method. The authors reported an "average erythema score" of 1 (corresponding to "very slight erythema (barely perceptible)" according to the Draize scale) at both 24 and 72 hours for both intact and abraded skin; however, the scores for individual animals were not provided, and it is therefore unclear how many of the animals displayed grade 1 reactions. No edema was observed. Although the authors stated that this study was conducted in general compliance with OECD guideline 404, there are several limitations. First, a DEP control was not included, and it is therefore impossible to distinguish whether slight erythema reactions were caused by this non-HHCB component of Galaxolide 50 solution. Additionally, it is unclear from the report whether residual chemical was removed after 24 hours, which could have led to prolonged exposure. Finally, the authors neglected to measure reversibility of the effects 14 days after patch removal, which is recommended by the guideline.

In the second dermal irritation/corrosion study in rabbits by IFF (1975a), albino rabbits (sex and strain/stock not specified; n = 3 per treatment group) were exposed to 25% Galaxolide 50 (final concentration of 17.5% HHCB in 7.5% DEP and 75% alcohol SDA 39C) on intact and abraded skin for 24 hours under occlusive dressing. Immediately after 24 hours and again 48 hours later, skin was evaluated according to the Draize method. No erythema or edema was observed at 24 and 72 hours. The authors stated that this study was conducted in general compliance with OECD guideline 404.

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Reference	Study Description	Effects	Study Quality Rating
(IFF, 2016a)	SD rats (n = 5 per sex per treatment group) exposed to 2,000 mg/kg undiluted Galaxolide (100% HHCB) or water on intact skin for 24 hours. OECD 402	"Slight erythema" (scores not provided) in all HHCB-treated females, but no males, between days 2 and 5; No edema	High
(IFF, 1973b)	Albino rabbits (sex and strain not specified; n = 3 per treatment group) exposed to Galaxolide 50 (final concentration of 70% HHCB in DEP) on intact and abraded skin for 24 hours. After 24 hours and again 48 hours later, skin was evaluated according to the Draize method. OECD 404	Average erythema score of 1 ("very slight (barely perceptible)") at 24 and 72 hours; No edema	Medium
(IFF, 1975a)	Albino rabbits (sex and strain not specified; n = 3 per treatment group) exposed to 25% Galaxolide 50 (final concentration of 17.5% HHCB in DEP and alcohol SDA 39C) on intact and abraded skin for 24 hours. After 24 hours and again 48 hours later, skin was evaluated according to the Draize method. OECD 404	No erythema or edema at 24 and 72 hours	Medium
DEP = diethyl phthalate; SD = Sprague-Dawley (rats)			

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2.3.2.3.5 Evidence Integration Summary

The available studies in humans and laboratory animals provide robust evidence that HHCB is not a skin irritant in humans. Specifically, HHCB did not produce any irritation in a sample of over 40 human subjects over nine repeated 24 hour exposures (IFF, 1973c) . Consistently, 70% HHCB (other constituents unknown) did not produce irritation in an OECD 439 *in vitro* study using a reconstructed human epidermis model (IFF, 2020b). Notably, according to the test guideline (pg. 1), this method "may be used to determine the skin irritancy of test substances as a stand-alone replacement test for *in vivo* skin irritation testing" given that "the reconstructed human epidermis closely mimics the biochemical and physiological properties of the upper parts of the human skin."

Findings of mild irritation in laboratory animals and in additional HIRPTs were not sufficient to warrant classification of HHCB as a skin irritant. Specifically, one acute dermal toxicity test examined the effects of neat HHCB and qualitatively reported "slight erythema" in five female rats, but no male rats, between 2 and 5 days after patch removal (IFF, 2016a). Additional dermal irritation/corrosion studies in laboratory animals and additional HRIPTs -tested mixtures of HHCB, either in alcohol SDA 39C or in DEP, which are known to cause irritation. These studies reported slight, often reversible erythema reactions that were likely related to the diluent rather than to HHCB. Specifically, two additional HRIPT studies testing HHCB diluted in alcohol SDA 39C observed reversible Grade 1 ("slight") or Grade 2 ("moderate") erythema in a subset of subjects that increased across these two studies in proportion to the amount of alcohol SDA 39C present, rather than the amount of HHCB present (1/40 subjects treated with 50% HHCB and 9/43 subjects treated with 3.75% HHCB, respectively). Data from an alcohol SDA 39C control group used in (IFF, 1975b) and data from a separate HIRPT study testing alcohol SDA 39C using occlusive vs. non-occlusive patches (IFF, 1963) indicate that these effects were either related to the alcohol SDA 39C diluent in the case of (IFF, 1975b) and/or due to the use of occlusive rather than semi-occlusive patches in some subjects in the case of (IFF, 1963). Similarly, an acute dermal irritation/corrosion study in rabbits observed Grade 1 ("slight") erythema in animals exposed to a mixture of HHCB and DEP as late as 72 hours after patch administration (IFF, 1973b). This was likely due to DEP, which has been shown to cause mild irritation in laboratory animals (Api, 2001).

Given the lack of effects across studies that tested neat HHCB in human subjects and in reconstructed human epidermis, EPA concluded that HHCB is not a skin irritant. This conclusion is also consistent with other regulatory agencies. Specifically, U.S. EPA (OCSPP, 2014) concluded that "HHCB showed no potential for skin irritation"; NICNAS (2019) concluded that "irritation effects are not sufficient to warrant hazard classification" ; and EU (ECB, 2008a, b) concluded that "based on the available data, HHCB is judged not to be a skin irritant."

2.3.2.4 Dermal Sensitization

2.3.2.4.1 Human Evidence

Available studies evaluating the skin sensitization potential of HHCB in humans include three human repeated insult patch tests (HRIPTs) (IFF, 1973c, d, 1964) and two studies involving patch tests in fragrance-sensitive individuals (An et al., 2005; Larsen et al., 2001). These studies are summarized in Table 2-6 and are discussed below.

Studies in the General Population

The sensitization potential of HHCB was tested during three human HRIPT studies (IFF, 1973c, d, 1964). In one study, 42 subjects (40 female and 2 male) were patch-tested with 100% neat HHCB under semi-occlusive dressing. In a second study, 43 subjects (36 female and 7 male) were patch-tested with 50% HHCB diluted in alcohol SDA 39C and with vehicle control under semi-occlusive dressing. In a third study, 40 subjects (28 female and 12 male) were patch-tested with 3.75% HHCB diluted in alcohol SDA 39C under semi-occlusive dressing, with the exception of 4 subjects that received occlusive dressings for the first three applications. In the induction phase each study, patches were applied to the upper arms for 24 hours for a total of nine applications. Subjects were instructed to remove the patches 24 hours after application, and skin was evaluated according to the Draize method on the day of the next scheduled application. Two weeks after removal of the final patch, challenge duplicate patches were applied, one to the original site and one to a fresh skin site. Subjects were instructed to remove the patches 24 hours after application, and skin was evaluated according to the Draize method. The schedule of when patches were applied and scored for each subject was redacted from two of the studies; however, the remaining study specifies that induction patches were applied 3 times per week for 3 weeks, and that 2 weeks after removal of the final patch, duplicate challenge patches were applied. No participants were sensitized across all three studies.

The following additional human studies are currently unavailable to EPA. Below are quoted summaries that appear in RIFM's assessment for HHCB (Api et al., 2023). These are also summarized in Table 2-6:

- "In a human maximization test, no skin reactions were observed when 10350 µg/cm2 HHCB was used."
- "In 2 additional CNIHs, conducted at 12,719 µg/cm^2 and 38760 µg/cm^2, no sensitization reactions were observed in 110 and 114 volunteer subjects, respectively (RIFM, 2021a; RIFM, 2021b)."

Studies in Fragrance Sensitive Populations

Larsen et al. (2001) tested the sensitization potential of HHCB in fragrance sensitive subjects across 8 centers in Japan, Northern Ireland, United States, England, Switzerland and Sweden. In this study, 178 fragrance sensitive subjects (sex not specified) were patch tested with 7% HHCB diluted in petrolatum. The challenge concentrations for the fragrance ingredients used for this study were established by testing 20 control subjects without clinical evidence of fragrance allergy to prove that the challenge concentrations for each individual ingredient were subirritant, using serial dilution challenge tests. Patch test readings were scored 2 to 3 days after exposure, and again 2 to 5 days after the first reading. A total of 3.4% (6 out of 178) of HHCB-treated participants scored positive for contact dermatitis. The study does not mention whether the vehicle was tested, and therefore, it is impossible to distinguish whether the low percentage of positive cases were due to HHCB or incidental. This study failed to report several additional details, making it impossible to verify the results. For example, it is unclear whether occlusive or semi-occlusive patches were used, it is unclear when patches were removed, the criteria for assessing a positive response were not included, and results were summary-level (*i.e.,* reported as the total number of individuals with a positive response without providing any data on individual reaction scores for the control or treatment groups).

An et al. (2005) tested the sensitization potential of the HHCB mixture Galaxolide 50 in fragrance sensitive subjects across 9 dermatology departments of university hospitals in Korea. In this study, 422 patients with suspected contact allergy who visited the hospitals over the period April 2002 to June 2003 were patch tested with 5% Galaxolide 50 diluted in petrolatum. No information was provided regarding the percentage of HHCB in the original Galaxolide-50 solution, as well as the identity of the diluent present in Galaxolide 50 (often DEP but not specified). A total of 1.2% (5 out of 422) of Galaxolide 50-

treated participants scored positive for contact dermatitis. All subjects were also patch tested with the petrolatum vehicle, which caused no sensitization in any of the subjects. Importantly, no control group was provided to isolate the effects of HHCB from other components of the original Galaxolide-50 solution; therefore, it is impossible to distinguish whether the low percentage of positive cases were due to HHCB, specifically. This study failed to report several additional details, making it impossible to verify the results. For example, it is unclear whether occlusive or semi-occlusive patches were used, it is unclear when patches were removed and when/how often readings were scored, the criteria for assessing a positive response were not included, and results were summary-level (*i.e.,* reported as the total number of individuals with a positive response without providing any data on individual reaction scores for the control or treatment groups).

Table 2-6. Summary of HHCB Studies Evaluating Effects on Dermal Sensitization in Humans

Reference	Study Description	Effects	Data Quality Evaluation Rating
(IFF, 1973c)	42 subjects (40 female and 2 male) were patch-tested with 100% neat Galaxolide. Patches were applied to the upper arms for 24 hours 3 times per week for 3 weeks. 2 weeks after removal of the final patch, challenge duplicate patches were applied, 1 to the original site and 1 to a fresh skin site.	No participants were sensitized	Acceptable/Non-guideline[a]
(IFF, 1973d)	43 subjects (36 female and 7 male) were patch-tested with 50% Galaxolide diluted in alcohol SDA 39C and with vehicle control. Patches were applied to the upper arms for 24 hours 3 times per week for 3 weeks. 2 weeks after removal of the final patch, challenge duplicate patches were applied, 1 to the original site and 1 to a fresh skin site.	No participants were sensitized	Acceptable/Non-guideline[a]
(IFF, 1964)	40 subjects (28 female and 12 male) were patch tested with 3.75% Galaxolide diluted in alcohol SDA 39C. Patches were applied to the upper arms for 24 hours 3 times per week for 3 weeks. After a 2-week rest period, a 24-hour challenge patch was made on a site previously not exposed.	No participants were sensitized	Acceptable/Non-guideline[a]
(Api et al., 2023) [b]	Human maximization test using 10,350 µg/cm^2 HHCB in an unknown number of subjects.	No participants were sensitized	N/A
(Api et al., 2023) [b]	Confirmation of no induction study in 110 subjects using 12,719 µg/cm^2 HHCB	No participants were sensitized	N/A
(Api et al., 2023) [b]	Confirmation of no induction study in 114 subjects using 38,760 µg/cm^2 HHCB	No participants were sensitized	N/A
(Larsen et al., 2001)	178 fragrance sensitive subjects (sex not specified) were patch tested with 7% HHCB diluted in petrolatum over a 3-month period. Patch test readings were scored 2–3 days after exposure, and again 2–5 days after the first reading.	3.4% (6 of 178) of participants scored positive for contact dermatitis	Low
(An et al., 2005)	422 patients with suspected fragrance allergy (352 female) were patch tested with 5% Galaxolide 50 diluted in petrolatum (percentage of HHCB not reported) over an unspecified period (duration of the entire study was over 1 year). Patch test readings were scored at unspecified times.	1.2% (5 of 422) participants showed skin responses to HHCB	Low

[a] References evaluated using the OPP DER format.
[b] Study not available to EPA but is summarized in other assessments.

2.3.2.4.2 Laboratory Animal and Mechanistic Evidence

EPA did not identify any reasonably available sensitization studies in laboratory animals for any exposure route. In their previous assessment, RIFM stated that "Mixed results were obtained from guinea pig maximization tests. It was predicted to be a sensitizer in one study (RIFM, 1981), whereas it was predicted not to be a sensitizer in another study (RIFM, 1996c)" (Api et al., 2023). This is unsurprising given that false positive reactions have been reported to occur for this assay (Basketter et al., 1998). Furthermore, a test method evaluation report by The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) provides quantitative evidence to support the limited predictive accuracy of the guinea pig maximization test for known human dermal sensitizers (NTP, 2011). Specifically, for 56 substances that had local lymph node assay (LLNA), guinea pig (*i.e.,* the guinea pig maximization test and/or the Buehler test), and human skin sensitization data, the overall correct classification rate of human sensitizers and nonsensitizers was 59% (33/56) for guinea pig tests (including the guinea pig maximization test and/or Buehler test). This was further broken down into rates of 57% for strong human sensitizers and 52% for other human sensitizers. Consequently, the lack of animal data does not decrease EPA's confidence in its conclusions regarding whether HHCB is a skin sensitizer.

HHCB is not predicted to react with skin proteins directly according to *in silico* modeling data from OECD QSAR Toolbox (version 4.2), Chemtunes ToxGPS (version 1.2), and Toxtree (version 3.1.0) (Api et al., 2023; NICNAS, 2019). EPA did not identify any additional information to provide any mechanistic support for sensitizing effects of HHCB.

2.3.2.4.3 Evidence Integration Summary

The available studies in humans provide robust evidence that HHCB is not a skin sensitizer. Specifically, three HRIPT studies found no effects across concentrations of 3.75, 50, and 100% HHCB (IFF, 1973c, d, 1964). Two additional patch test studies, which reported positive reactions in low percentages (1.2 and 3.4%) of patients that were already sensitized to fragrance materials, were uninformative, either due to lack of a vehicle control with which to compare a normal incidence of reactions in these sensitive populations, or because a mixture of HHCB with other, unnamed chemicals was tested; therefore, the effects of HHCB could not be distinguished other components (An et al., 2005; Larsen et al., 2001). Given the limitations of these two studies and given the negative results reported in three studies that tested higher concentrations of HHCB, EPA concluded that these findings are not sufficient to warrant classification of HHCB as a skin sensitizer.

In contrast, RIFM concluded in its 2023 assessment that "based on the weight of evidence from structural analysis and animal and human studies, HHCB is a sensitizer with a weight of evidence no expected sensitization induction level (NESIL) of 38000 µg/cm^2" (Api et al., 2023). Notably, this NESIL is orders of magnitude larger than the highest dermal exposure values reported in Appendix C of the *Draft Human Exposure Assessment for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (U.S. EPA, 2026h); therefore, EPA does not expect that sensitization will occur at doses relative to human exposure. Furthermore, RIFM's conclusion appears to be based on a single, limited patch test in fragrance-sensitive individuals discussed above (An et al., 2005) and on equivocal evidence from two guinea pig maximization tests that are unavailable to EPA. HHCB is not predicted to react with skin proteins directly according to *silico* modeling data and the RIFM assessment does not include any opposing structural analysis data.

In summary, while two limited human patch tests report positive reactions in fragrance sensitive populations, three HRIPTs report negative skin sensitization at higher doses (up to 100% HHCB).

Animal evidence from guinea pig maximization tests is equivocal, which is unsurprising given that this method has been demonstrated to have limited (nearly 50%) accuracy in predicting human outcomes (NTP, 2011). Based on this weight of evidence, EPA concludes that HHCB is not a sensitizer. This conclusion is consistent with other regulatory agencies. Specifically, U.S. EPA (OCSPP, 2014) concluded that "HHCB showed no potential for sensitization," and NICNAS (2019) and EU (ECB, 2008a, b) concluded that HHCB "is not a sensitizer."

2.3.2.5 Weight of Scientific Evidence Conclusions on Dermal Hazard

EPA considered the weight of scientific evidence across dermal absorption data and dermal hazard endpoints including acute and subchronic dermal toxicity, dermal irritation, and dermal sensitization for HHCB. The Agency concluded that dermal absorption is anticipated to be low, and that there is robust evidence in humans and animals that HHCB is not hazardous via the dermal route. Specifically, dermal absorption was shown to have a lower bound of less than 0.1% based on a study in human subjects, and an upper bound of 5.2 to 8.1% based on studies in human skin samples. When applied neat to the skin, HHCB did not produce any systemic dermal effects at doses as high as 1,000 mg/kg in an acute dermal toxicity test, did not produce irritation in an *in vitro* OECD 439 study, and did not produce irritation or sensitization in an HRIPT.

EPA additionally considered evidence from studies involving mixtures of HHCB. HHCB did not produce any systemic dermal effects in subchronic dermal toxicity studies at doses as high as 130 mg/kg-day. Mild, reversible irritation was observed in acute irritation/corrosion tests and in HRIPTs and was likely due to the presence of alcohol SDA 39C (a GHS category 2 skin irritant) and/or DEP, which produces mild skin irritation in animals. HHCB did not produce dermal sensitization in two HRIPT studies evaluating mixtures in alcohol SDA 39C. While two studies found evidence of sensitization in a small number of fragrance sensitive subjects patch tested with final HHCB concentrations of 7% and less than 5%, the validity of these findings is uncertain due to study limitations and given two negative HRIPTs testing HHCB at concentrations of 3.75 and 50%.

In summary, EPA determined that HHCB is poorly absorbed and is not hazardous via the dermal route of exposure. The Agency's conclusions regarding dermal absorption, acute dermal toxicity, dermal irritation, and dermal sensitization are consistent with other regulatory agencies (NICNAS, 2019; ECB, 2008a, b). Therefore, although dermal exposures are estimated in Appendix C of the *Draft Human Exposure Assessment for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (U.S. EPA, 2026h), EPA did not derive a dermal hazard value or estimate risks in the Draft Risk Evaluation for HHCB (U.S. EPA, 2026i).

2.3.3 Oral Route

For the oral route, EPA is focusing its hazard identification on developmental and reproductive toxicity. This includes all endpoints that were taken forward for risk characterization by previous existing risk assessments (OCSPP, 2014; ECB, 2008a, b). This also includes all endpoints for which updated hazard information was identified through systematic review that was not considered by previous assessments. In the following sections, EPA assessed human, laboratory animal, and mechanistic data and integrated these evidence streams to independently make conclusions based on the weight of scientific evidence for each hazard outcome. Hazard outcomes with sufficient confidence and quantitative study data then underwent dose-response analysis (Section 2.4). Additional human health hazard outcomes from oral exposure that were not considered for dose-response are discussed in Appendix C.

Previous assessments concluded that HHCB has "an overall low concern for developmental toxicity" (OCSPP, 2014) or "no specific reproductive or developmental toxicity" (NICNAS, 2019). No multigenerational reproductive toxicity studies were available at the time of these assessments, and these conclusions were based on developmental and reproductive toxicity information from a 90-day repeated-dose oral toxicity study (reproductive organ data) (Api and Ford, 1999), a prenatal developmental toxicity study report that contains insufficient detail (Christian et al., 1999; Argus Research Labs, 1997), and a peri-natal reproductive toxicity study that is only available to EPA in the form of a conference abstract (Ford and Bottomley, 1997). Since these assessments, the Agency identified a new EOGRT study in rats (IFF, 2021). Because the EOGRT study addresses a key uncertainty referenced in EPA's previous assessment by providing a multigenerational reproductive toxicity study for HHCB (OCSPP, 2014), EPA reevaluated the weight of evidence for developmental and reproductive toxicity considering the EOGRT and other newly available studies.

2.3.3.1 Human Evidence

EPA did not identify any epidemiologic studies evaluating developmental or reproductive effects for HHCB.

2.3.3.2 Laboratory Animal Evidence

Studies evaluating the effects of HHCB exposure on developmental and reproductive toxicity in laboratory animals include one non-guideline prenatal developmental toxicity study in rats (Christian et al., 1999; Argus Research Labs, 1997); one perinatal reproductive toxicity study in rats (Ford and Bottomley, 1997); OECD 408 one repeated-dose 90-day oral toxicity study in rats that evaluated reproductive organ data (Api and Ford, 1999); and one uterotrophic assay in mice (Seinen et al., 1999). In addition to these studies, EPA identified five studies that were not considered in previous assessments. These include an OECD 443 EOGRT study (IFF, 2021) and the associated range-finding study (IFF, 2020a) in rats, one OECD 414 prenatal developmental toxicity study in rabbits (IFF, Date Unknown-b), and two non-guideline studies assessing androgen disruption in male rats exposed via intraperitoneal injection (Li and Wang, 2023; Li et al., 2023). These studies are summarized in Table 2-7 and are discussed below.

2.3.3.2.1 Prenatal Developmental Toxicity Studies

In an OECD 414 prenatal developmental toxicity study (IFF, Date Unknown-b), pregnant New Zealand White rabbits (n = 22 per dose) were exposed to 0, 10, 30, or 100 mg/kg-day HHCB via gavage on gestation day (GD) 6 to 28. Dams in the highest dose group (100 mg/kg-day) showed statistically significant decreases in mean body weight gain (11%) and food consumption (5%) during GD 6 to 29; however, there was no clear dose-response relationship because these outcomes increased significantly relative to control at lower doses. The following outcomes related to maternal and developmental toxicity were measured and reported as unchanged relative to the vehicle control. In dams, clinical signs; gravid uterus weight; implantation sites; pre-implantation loss; post-implantation loss; pregnancy incidence; and mean live litter size remained unchanged. In fetuses obtained on GD 29, no effects of HHCB exposure on body weight, sex ratio, and incidences of external, visceral, or skeletal malformations were observed. Although the study authors described the study as having been conducted in general compliance with OECD guideline No. 414, there were several limitations that made the data useful for weight of evidence, but inappropriate for dose-response. Specifically, the study authors reported that there was vehicle-related toxicity (reduced food consumption, reduced fecal output, and reduced body weight gain) in dams relative to historic control data; however, the validity of this comparison is uncertain because no information was provided on whether the historic control data were recent or gathered from the same laboratory. Additionally, the authors stated that they performed pairwise comparisons using two-sided tests that were reported at the 1 and 5% levels; however, it is

unclear if any changes were statistically significant because statistical significance was not denoted in any data tables.

In a second prenatal developmental toxicity study that was non-guideline (Christian et al., 1999; Argus Research Labs, 1997), pregnant SD rats (n = 25 per dose) were exposed to 0, 50, 150, or 500 mg/kg-day HHCB via gavage on GD 7 to 17. Maternal toxicity was noted in dams starting at 150 mg/kg-day. Specifically, body weight gain (percentage unknown) and food consumption (percentage unknown) decreased dose-dependently during GD 7 to 18 and reached statistical significance compared to vehicle control starting at 150 mg/kg-day. Additionally, in dams in the high dose group (500 mg/kg-day), body weight decreased during GD 8 to 20 (percentage unknown), and excess salivation, urine-stained abdominal fur, red or brown substance on the forepaws, and alopecia in 4 to 9 of 25 rats was noted. In fetuses, the following outcomes were changed relative to vehicle control at the highest tested dose (500 mg/kg-day): Decreased live fetal body weight that was statistically significant compared to vehicle control but that fell within historical control ranges and increased incidences of axial skeletal (vertebral/rib) malformations in three fetuses that were not litter mates that were not statistically significant. These malformations included hemivertebrae, associated unilateral or bifid ossification of vertebral centra and/or fused vertebral arches and centra/ribs. Although they were described as "not spontaneous in origin," incidence data was not provided in the study for vehicle controls or for historical controls; therefore, this conclusion could not be verified.

The following outcomes that were evaluated were unaffected by HHCB treatment: pregnancy rates, abortions, premature deliveries, dam mortality, litter averages for corpora lutea, implantations, live fetuses, resorption sites, post implantation loss, litter size, and number of live fetuses per litter. Although the study authors reported that this study was conducted in general compliance with OECD guideline No. 414, HHCB exposure began after GD5 (when implantation typically occurs in the rat) and was therefore not compliant with the OECD guideline. Additionally, the results were qualitatively described, and the raw data were not provided; therefore, the data are useful for weight of evidence but uninformative for dose-response.

2.3.3.2.2 Studies in Perinatally-Exposed Rats

In a study conducted according to the ICH Guideline on the Detection of Toxicity to Reproduction for Medicinal Products with slight modifications (Ford and Bottomley, 1997), pregnant SD rats (n = 28 per dose) were exposed to 0, 2, 6, or 20 mg/kg-day HHCB via gavage from GD 14 through PND 21. Offspring received normal diets after weaning and were allowed to produce the F2 generation after 84 days of age. EPA did not have access to the primary data; therefore, the information described herein is taken from the 2008 assessment conducted by the EU (ECB, 2008a, b). No treatment-related effects were found on any of the endpoints evaluated. In F1 animals, these included sex, body weight, and external abnormalities after parturition; developmental milestones during pre-weaning (*i.e.,* surface righting reflex, startle reflex, air righting reflex and pupil reflex); neurological parameters in sexually mature animals (*i.e.,* motor coordination and balance, activity and avoidance); and reproductive parameters in adults (*i.e.,* time of pregnancy, estrous cyclicity, pre-coital time, pregnancy rates, and duration of gestation). In F2 animals, there were no external abnormalities from parturition to PND 21; however, the outcomes measured in F2 animals are unclear from the study summary provided by the EU (ECB, 2008a, b). Given that EPA only has access to qualitative descriptions of the study from other assessments, this study is uninformative for dose-response.

In a simplified reproductive/developmental toxicity screening test (modified OECD 421) that was used as a range-finding study for the EOGRT study, male and female Wistar rats (n = 10 per sex per dose) were exposed to HHCB via the diet at achieved concentrations of 34/38 and 121/134 mg/kg-day HHCB

in males/females (IFF, 2020a). Males were dosed for 29 days (*i.e.,* 2 weeks prior to mating, during mating, and up to the day of necropsy). Females were dosed 2 weeks prior to mating, during mating, gestation, and lactation, and up to the day of necropsy (*i.e.,* on lactation day [LD] 21–23 for females that delivered). Notably, this study did not include a concurrent control group; instead, the results were compared to historical control data. The following results relevant to developmental and reproductive toxicity were noted in F1 pups: Starting at the low dose group (34/38 mg/kg-day), body weight on PND 1 decreased dose-dependently (8.7/8.8 to 11.7/9.9% in males/females). At the highest tested dose (121/134 mg/kg-day), body weight on PND 21 decreased 11.1/9.2% in males/females. The following outcomes were unaffected by HHCB treatment: mortality; clinical signs; F0 body weight; F0 food consumption; estrous cycle data; mating performance and fertility; sperm parameters; stillbirths; duration of gestation; pre-birth loss; pup viability; litter size; pup observations; organ weights other than thyroid, liver, and kidney; and macroscopic observations in all organs. Histology was not evaluated in any tissues, including reproductive tissues, except for the kidney. Ultimately, this study is uninformative for dose-response due to limitations including a lack of statistical comparisons and no concurrent study control.

The design and results of the following study are described in more detail in *Draft Data Evaluation Records for Human Health Hazard for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (U.S. EPA, 2026b). Briefly, in an OECD 443 EOGRT study (IFF, 2021), male and female Wistar rats (n = 25 per sex per dose) were exposed to 0, 470, 825, or 1,650 ppm HHCB in the diet from 10 weeks prior to mating in F0 animals continuously throughout gestation and lactation. In F0 males, this corresponded to average intake of 25.8/45.9/94.1 mg/kg-day at the low/medium/high doses, respectively. In F0 females, average intake ranged from 26.8 to 34.4/45.6 to 57.5/91.7 to 116.3 mg/kg-day at the low/medium/high doses depending on pre-mating vs. gestation vs. lactation. At weaning, the F1 pups were divided into Cohorts 1A, 1B, and 1C (containing 20 pups/sex/treatment level/cohort) and fed the same dietary concentrations as their parents. In F1 males, this corresponded to an average intake of 33.9/59.6/123.3 mg/kg-day at the low/medium/high doses. In F1 females, this corresponded to average intake of 27.4 to 35/47.6 to 60/95.2 to 122.1 mg/kg-day at the low/medium/high doses depending on pre-mating vs. gestation vs. lactation. Cohort 1A animals were assessed for clinical pathology, time between vaginal patency and onset of estrus, estrous cycle data, differential ovarian follicle counts, sperm parameters, and splenic lymphocyte subpopulation analysis, and were terminated on PND 85 to 93. Cohort 1B offspring were mated to produce F2 litters, assessed for reproductive parameters, and terminated after mating (males) or on LD 21 to 23 (females). F1 offspring from all cohorts were assessed for vaginal opening and preputial separation, and Cohort 1C animals were terminated after positive identification of these landmarks. Some unselected F1 offspring were assigned to a surplus cohort for assessment of thyroid-related hormones and organ weights (on PND 21), with termination on PND 21 to 23. Selected culled PND 4 pups were also used for hormone assessment. In addition to standard assessment of litter parameters for F1 and F2 offspring, F2 pups were also examined for AGD on PND 1 and were terminated on PND 21 to 23.

There were no treatment-related effects on body weight, body weight gain, or food consumption in the F0 males or females at any treatment level. There were no adverse, treatment-related effects on mortality; clinical signs of toxicity; hematology, clinical chemistry, and urinalysis parameters; splenic lymphocyte subpopulations; and macroscopic pathology in the F0 and/or F1 adult animals. There were no adverse, treatment-related effects on viability; clinical signs of toxicity; TSH concentrations; and macroscopic pathology in F1 and/or F2 animals. Additionally, there were no adverse, treatment-related effects on the following endpoints related to reproductive toxicity: estrous cycle parameters in the F0 and Cohort 1A/1B females; sperm parameters in the F0 or Cohort 1A males; mating/fertility indices, pre-coital interval, gestation index in the F0 or Cohort 1B animals; or differential ovarian follicle count

evaluations in the Cohort 1A females. Developmental neurotoxicity and immunotoxicity endpoints were not investigated, and although these assessments are optional according to the test guideline, the bases for not including these endpoints was not provided in the study report.

As discussed in the *Draft Data Evaluation Records for Human Health Hazard for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (U.S. EPA, 2026b), the developmental/reproductive outcomes that were considered biologically adverse and related to HHCB treatment included reduced F1 and F2 offspring body weight from PND 1 to 21 that persisted through adulthood in F1 animals (summarized in Tables 3a-d and 10a-b of the DER for this study) (U.S. EPA, 2026b). Additionally, preputial separation was significantly delayed in F1 males (summarized in Table 12 of the DER) and AGD significantly decreased in F2 (but not F1) males and females at PND 1 (summarized in Table 11 of the DER). These effects are discussed in more detail below.

Decreased Body Weight in F1 and F2 Pups (PND 1–21): Adverse (*i.e.,* ≥5%), treatment-related decreases in F1 and F2 pup body weight reached statistical significance starting at the middle dose group (achieved F0 and F1 maternal dose of 45.6 and 47.6 mg/kg-day during gestation) at early postnatal days and persisted through PND 21. Specifically, in F1 males, dose-dependent decreases in body weight reached significance in the middle (6%) and high (9%) dose groups on PND 1; by PND 21, body weights were still decreased significantly in the middle (6%) and high (11%) dose groups. Similarly, in F1 females, dose-dependent decreases in body weight reached significance in the middle (5%) and high (8%) dose groups on PND 7; by PND 21, body weights were still decreased significantly in the middle (5%) and high (11%) dose groups. Male and female weight gains decreased dose-dependently and significantly during all tested timeframes (PND 1–4; 4–7; 7–14; 14–21; 1–21), with the largest decrease at PND 4 to 7 in F1 males (14%) and at PND 7 to 14 in F1 females (15%) at the high dose group.

In F2 males, dose-dependent decreases in body weight reached significance at the middle (−5%) and high (5%) dose groups on PND 4; by PND 21, body weights remained significantly decreased in the middle (7%) and high (12%) dose groups. In F2 females, dose-dependent decreases in body weight reached significance in the middle (7%) and high (12%) dose groups on PND 14; by PND 21, body weights remained significantly decreased in the middle (6%) and high (9%) dose groups. Male and female body weight gains decreased dose-dependently and significantly during all tested timeframes (PND 1–4; 4–7; 7–14; 14–21; 1–21), with the largest change at PND 7 to 14 (−19% in F1 males and females) at the high dose group.

Decreased Body Weight in F1 Males (Post-Weaning Treatment Days 1–120): Body weight continued to decrease dose-dependently in F1 males from post-weaning treatment days 1 to 120, and this occurred alongside decreased food consumption. These changes were considered adverse (*i.e.,* ≥10%) starting at the middle dose group (achieved F0 maternal dose of 45.6 mg/kg-day during gestation). Specifically, from day 1 to 120, statistically significant decreases in body weight continued to grow from 5 to 8%, 6 to 11%, and 11 to 17% in the low, middle, and high dose groups, respectively. Overall body weight gains (days 1–120) decreased significantly by 8, 12, and 18% in the low, middle, and high dose groups, respectively. Additionally, overall food consumption significantly decreased by 8, 12, and 18%.

Decreased Body Weight in F1 Females (Premating, Gestation, and Lactation): By pre-mating day 72, body weight decreases, decreases in overall body weight gain, and changes in overall food consumption were less than 10% in F1 females and were not considered adverse— though they were dose-dependent and statistically significant. During gestation (GD 0–10), statistically significant differences were considered adverse in the high dose group (achieved maternal F0 dose of 91.7 mg/kg-day during gestation) based on body weights that were 9 to 11% lower relative to control on GD 0 and 20,

decreased overall body weight gain of 16%, and decreased overall food consumption of 12%. During lactation, changes in body weight, body weight gain, and food consumption recovered to less than 10% relative to control and were not considered adverse.

Other Developmental and Reproductive Outcomes in F1 and F2 Pups (PND 1–21): In F1 males, preputial separation was delayed significantly by approximately 1.4 to 2.5 days at all treatment levels in a manner unrelated to dose, with the shortest delay in the high dose group (achieved dose of 91.7/95.2 mg/kg-day during gestation in F0/F1 dams). The delay at the highest dose group was considered adverse, as it occurred in conjunction with decreased body weight in these animals. Additionally, AGD decreased significantly and dose-dependently in F2 (but not F1) males and females starting at the middle dose group (achieved dose of 45.6/47.6 mg/kg-day during gestation in F0/F1 dams). Specifically, AGD decreased 16 and 21% relative to controls in males and 22 and 26% in females in the middle and high dose groups, respectively.

2.3.3.2.3 Subchronic Toxicity Studies Evaluating Reproductive Parameters

In an OECD 408 repeated-dose 90-day oral toxicity study (Api and Ford, 1999), male and female Sprague-Dawley rats (n = 15 per sex per dose) were exposed to 0, 5, 15, 50, or 150 mg/kg-day HHCB in the diet. Except for increased relative liver weights in males (discussed in more detail in Appendix 3.4.3C.2.1), no treatment-related effects were found on any of the endpoints assessed. These endpoints included mortality; clinical signs; body weight; food consumption; hematology and blood chemistry changes; weights of reproductive organs (ovaries and testes); and histopathology in the ovaries, testes, epididymides, mammary gland, prostate, seminal vesicles, uterus, and vagina.

2.3.3.2.4 Studies Evaluating Potential for Endocrine Disruption

Available *in vitro* studies indicate that HHCB acts as a weak agonist and antagonist for both the estrogen (ER) and androgen receptors (AR). Specifically, HHCB showed weak (*i.e.,* orders of magnitude lower than estradiol [E2]) or no ER alpha agonist activity and no ER beta agonist activity in yeast or in human cell lines transfected with human ER gene reporters (HEK 293, U2-OS, HeLa, and MCF-7) (Cavanagh et al., 2018; Simmons et al., 2010; Gomez et al., 2005; Schreurs et al., 2005; Schreurs et al., 2002; Seinen et al., 1999). HHCB acted as an antagonist for ER alpha in yeast and in human HEK 293, and U2-OS cells, and for ER beta in HEK 293 and U2-OS cells (Cavanagh et al., 2018; Simmons et al., 2010; Schreurs et al., 2005; Schreurs et al., 2002). Notably, the anti-estrogenic activity of HHCB was shown to be weaker than the positive control ER antagonists SERM 4-hydroxytamoxifen, ICI 182,780, and 4-OH-tamoxifen (Schreurs et al., 2005; Schreurs et al., 2002). Regarding the AR, HHCB showed no AR agonist activity in U2-OS and PALM cell lines transfected with human androgen receptor gene reporters (Cavanagh et al., 2018; Schreurs et al., 2005); however, it did show AR agonist activity in HEK 293, although its transcriptional activity that was substantially weaker than that of testosterone (Li et al., 2023). HHCB showed antagonist activity for the AR in HEK 293 and PALM cell lines (Cavanagh et al., 2018; Simmons et al., 2010; Schreurs et al., 2005; Schreurs et al., 2002). The anti-androgenic activity of HHCB was also shown to be weaker than the positive control AR antagonists flutamide and vinclozolin (Schreurs et al., 2005) and bicalutamide (Cavanagh et al., 2018). HHCB was additionally an antagonist but not an agonist for the human progesterone receptor (PR) in U2-OS cells; however, HHCB was weaker than the positive control PR antagonist RU486 (Schreurs et al., 2005).

In a mouse uterotrophic assay (Seinen et al., 1999), 21-day old female Balb/c mice (n = 6 per dose) were exposed to 0, 6, or 40 mg/kg-day via the diet for 2 weeks. A positive control group received 140 µg 17-β estradiol on days 1, 5, 9, and 12 via subcutaneous injection. Animals in the positive control group had significantly higher uterine weights and lower thymus weights relative to control animals, while HHCB had no effects on body weight, uterus weight, or thymus weight. Notably, this study pre-dated the

relevant OECD 440 guideline, and it deviated from the appropriate weaning age and length of bioassay period recommended in the available method at the time published by Thigpen et al. (1987). Specifically, Thigpen et al. recommended that mice should be weaned at 15 days of age and that the bioassay period should be terminated at 7 days, when the mice are 22 days old, for best reproducible results.

In a non-guideline study assessing the androgenic activity of HHCB in immature male rats by Li et al. (Li et al., 2023), 3 to 4-week-old intact (non-castrated) male SD rats (n = 6 per treatment group) were exposed to 0, 5, 10, or 20 mg/kg-day HHCB via intraperitoneal injection for 7 days. HCCB was dissolved in DMSO and diluted in corn oil; therefore, control animals received DMSO diluted in corn oil. Relative seminal vesicle weight increased dose-dependently and significantly across all tested doses of HHCB (percentage not provided). Relative prostate weight increased (percentage not provided) across all tested doses, although the increase was not statistically significant in the middle dose group (10 mg/kg-day). No treatment-related effects were observed on relative testicle, kidney, or liver weights.

The following additional effects were only evaluated for a single dose group (20 mg/kg-day). Regarding histological changes in the male reproductive organs, increased folding of the prostate epithelial cells and a slight increase in the gap between adjacent seminiferous tubules were noted; however, data were not measured quantitatively nor statistically analyzed. No effects were observed on the following histopathology endpoints in the seminal vesicles: luminal volume and number and extension of epithelial folds. No effects were observed on the following histopathology endpoints in the ventral lobe of the prostate: lumen space, stroma, and lumen morphology. No effects were observed on the following histopathology endpoints in the testes: morphology and diameter of seminiferous tubules, and arrangement of spermatocytes in the seminiferous tubules. The proportion of MKI67-positive cells and of PCNA-positive cells, which are both markers of proliferation index, was significantly higher than in the control group in epithelial cells from both seminal vesicle and prostate tissue. Additionally, serum testosterone levels significantly increased, while luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels significantly decreased.

The same authors also evaluated the reproductive toxicity of HHCB in mature male rats (Li and Wang, 2023) in a non-guideline study. 8-9-week-old male Sprague-Dawley rats (n = 10 per treatment group) were exposed to 0 or 20 mg/kg-day HHCB via intraperitoneal injection for 30 days. HHCB was dissolved in DMSO (dimethyl sulfoxide), and the stock solution was diluted with corn oil. Control animals received DMSO diluted in corn oil. After 30 days of treatment, blood was collected from all animals for measurement of serum testosterone, gonadotropin-releasing hormone (GnRH), FSH, and LH. Organs (*i.e.,* testicles, seminal vesicles, prostate, epididymis, liver, and kidney) were collected for organ weights, sperm parameters, and histology. Evaluation of superoxide dismutase (SOD) and malondialdehyde (MDA) levels in testicles and transcriptomic analysis were also conducted, with results briefly summarized in Table A-1 of the study (Li et al., 2023).

The body weights of all animals were similar at the beginning of the study, and body weight gain did not change significantly over the course of the 30 days of treatment. Relative (but not absolute) seminal vesicle weight significantly increased in HHCB-treated animals. No treatment-related effects were observed on relative testes weight, prostate weight, kidney weight, or liver weight.

Sperm concentration and motility significantly decreased and abnormal sperm rate significantly increased in HHCB-treated animals relative to control. Additionally, serum testosterone levels decreased, and GnRH, FSH, and LH levels increased significantly in HHCB-treated animals relative to control.

Regarding histological changes, the average height of columnar epithelial cells in the seminal vesicles was significantly reduced, and the average thickness of smooth muscle layers was increased. There were no changes in the lumen area of the seminal vesicles. In the testes, the authors reported that the convoluted seminiferous tubules were loosely packed, the intertubular gap was increased, and the large vacuoles appeared in some convoluted seminiferous tubules in HHCB-treated rats; however, these results were not quantified nor statistically compared to control animals. Additionally, the diameter of the seminiferous tubules and the height of the seminiferous tubular epithelium were unchanged relative to control.

Table 2-7. Summary of HHCB Studies Evaluating Effects on Developmental and Reproductive Toxicity in Animals

Reference(s)	Study Description	NOAEL/LOAEL (mg/kg-day)	Effects	Study Quality Rating
Gestational exposure				
(IFF, Date Unknown-b)	New Zealand White rabbits (n = 22 per dose) were gavaged with 0, 10, 30, or 100 mg/kg-day during GD 6–28. Fetuses were obtained via caesarean section on GD 29. (OECD 414)	30/100 (maternal) 100/ND (developmental)	At 100 mg/kg-day: ↓ Dam body weight gain during GD 6–29 (11%); ↓ Food consumption during GD 6–29 (5%)	Uninformative
(Christian et al., 1999; Argus Research Labs, 1997)	SD rats (n = 25 per dose) were gavaged with 0, 50, 150, or 500 mg/kg-day on GD 7–17. Fetuses were obtained via cesarean section on GD 20.	50/150 (maternal) 150/500 (developmental)	At ≥ 150 mg/kg-day: ↓ Dam body weight gain (percentage not reported) during GD 7–18; ↓ Food consumption GD 7–18 (percentage not reported) At 500 mg/kg-day: *In dams:* ↓ Body weight GD 8–20 (percentage not reported); Excess salivation, urine-stained abdominal fur, red or brown substance on the forepaws and alopecia (4–9/25 rats) *In fetuses:* ↓ Fetal body weight (percentage not reported); ↑ Incidence of axial skeletal (vertebral/rib) malformations including hemivertebrae, associated unilateral or bifid ossification of vertebral centra and/or fused vertebral arches and centra/ribs; ↓ number of ossification sites in the metatarsals	Low
Perinatal exposure (includes multi-generational studies)				
(IFF, 2021) [a]	Male and female Wistar rats (n = 25 per sex per dose) were exposed to 0, 470, 825, or 1,650 ppm HHCB via the diet during 10 weeks prior to mating in F0 animals through PND	26.8/45.6	At ≥ 825 ppm: *In F1 animals:* ↓ Body weight and body weight gain in male and female pups (PND 1–21)	Acceptable/ Guideline[a]

Reference(s)	Study Description	NOAEL/LOAEL (mg/kg-day)	Effects	Study Quality Rating
	21–23 in F2 pups. Achieved doses in mg/kg-day in the low/medium/high dose groups were: In F0 males: 25.8/45.9/94/1 In F0 females: 26.8–34.4 / 45.6–57.5 / 91.7–116.3 In F1 males: 33.9/59.6/123.3 In F1 females: 27.4–35 / 47.6–60 / 95.2–122.1 mg/kg-day EOGRT study (OECD 443)		*In F2 animals:* ↓ Body weight and body weight gain in male and female pups (PND 1–21); ↓ Mean AGD in males and females (PND 1) At 1,650 ppm: *In F1 animals:* ↓ Body weight, body weight gain, and food consumption in dams during gestation; delayed preputial separation alongside decreased body weight at sexual maturation in males	
(IFF, 2020a)	Wistar rats (n = 10 per sex per dose) exposed via diet to 34/38 and 121/134 mg/kg-day for males/females. Males were dosed for 29 days (*i.e.,* 2 weeks prior to mating, during mating, and up to the day of necropsy). Females were dosed 2 weeks prior to mating, during mating, gestation, and lactation, and up to the day of necropsy (*i.e.,* on LD 21–23 for females that delivered). OECD 421	ND/34	At ≥ 34/38 mg/kg-day (males/females) relative to historical control: *In F1 pups:* ↓ body weight on PND 1 (8.7/8.8%-11.7/9.9 in males/females) At ≥ 121/134 mg/kg-day (males/females) relative to historical control: *In F1 pups:* ↓ body weight on PND 21 (11.1/9.2% in males/females)	High
(Ford and Bottomley, 1997)	Pregnant SD rats (n = 28/dose) were gavaged with 0, 2, 6, or 20 mg/kg/day HHCB from GD 14 through PND 21. F1offspring received normal diets after weaning and were allowed to produce the F2 generation after 84 days of age. ICH Guideline on the Detection of Toxicity to Reproduction for Medicinal Products	20/ND	No effects reported	N/A; EPA did not have access to this tech report. Available info is taken from the referenced conference abstract and from a summary in the EU assessment (ECB, 2008a, b).

Reference(s)	Study Description	NOAEL/LOAEL (mg/kg-day)	Effects	Study Quality Rating
Exposure post-weaning				
(Seinen et al., 1999)	Uterotrophic assay in 21-day old female Balb/c mice (n = 6 per dose) exposed via diet to 0, 6, or 40 mg/kg-day for 2 weeks.	40/ND	No effects reported	Acceptable/Non-guideline[a]
(Li et al., 2023)	3–4 week old male SD rats (n = 6 per dose) were exposed to HHCB for 7 days via intraperitoneal injection at doses of 0, 5, 10, 20 mg/kg-day	10/20	At ≥ 5 mg/kg-day: ↑ relative weight of seminal vesicles; ↑ relative prostate weight (n.s. at middle dose) At 20 mg/kg-day: The following effects were not assessed in any other dose groups: Qualitative histological changes in prostate (increased folding of epithelial cells and increased gap between seminiferous tubules); ↑MKI67-positive cells in seminal vesicles and prostate; ↑PCNA-positive cells in seminal vesicle and prostate; ↑ serum testosterone; ↓ serum FSH; ↓ serum LH	N/A; supplemental study
(Li and Wang, 2023)	8–9-week-old male SD rats (n = 10 per dose) were exposed to 0 or 20 mg/kg-day HHCB for 30 days via intraperitoneal injection	ND/20	At 20 mg/kg-day: ↑ Relative seminal vesicle weight; ↓ sperm concentration; ↓ sperm motility; ↑abnormal sperm rate; ↓ serum testosterone; ↑ serum GnRH, FSH, and LH; ↑MDA in serum and testes; ↓ SOD in serum and testes; ↓ average height of columnar epithelial cells in seminal vesicles; ↑ average thickness of smooth muscle layers in seminal vesicles; qualitative histopathological changes in the testes; ↓ expression of genes involved in steroid hormone biosynthesis (*Dhcr7, Cyp17a1, Srd5a2*); ↑ expression of genes involved in GnRH signaling (*Gnrhr, Fshr, Camk2d, Map3k2*); ↓ *Tex14* gene expression	N/A; supplemental study
(Api and Ford, 1999)	Male and female SD rats (n = 15 per sex per dose) were exposed to 0, 5,	150/ND	No effects reported	Medium

Reference(s)	Study Description	NOAEL/LOAEL (mg/kg-day)	Effects	Study Quality Rating
	15, 50, or 150 mg/kg-day HHCB in the diet for 90 days. OECD 408			

AGD = anogenital distance; *Camk2d* = calcium/calmodulin dependent protein kinase II delta; *Cyp17a1* = cytochrome P450 family 17 subfamily A member 1; FSHR = follicle stimulating hormone receptor; GD = gestational day; *Gnrhr* = gonadotropin releasing hormone receptor; *Fshr* = follicle stimulating hormone receptor; IP = intraperitoneal; LD = lactation day; LOAEL = Lowest-observed-adverse-effect level; *Map3k2* = mitogen-activated protein kinase 2; ND = not determined; NOAEL = no-observed-adverse-effect level; N.S. = not significant; OECD = Organization for Economic Co-operation and Development; PND = post-natal day; SD = Sprague-Dawley (rats); *Srd5a2* = steroid 5-alpha-reductase; *Tex14* = testis expressed 14

[a] Reference evaluated using the OPP DER format.

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2.3.3.2.5 Evidence Integration Summary

Potential for Endocrine Disruption

Androgen Disruption: The available studies show slight evidence that HHCB has weak anti-androgenic activity. Specifically, HHCB demonstrated activity as an AR agonist and antagonist *in vitro*, although activity was less potent than that of testosterone and of known positive control antagonists. *In vivo*, HHCB showed effects on a subset of androgen-sensitive endpoints that were measured. However, these findings are restricted to two non-guideline intraperitoneal injection studies that tested a single dose; they were not replicated across the OECD 443 EOGRT and OECD 408 90-day repeated dose oral toxicity studies that tested higher doses and more relevant (*i.e.,* oral) exposure routes. This is discussed below.

In vitro studies indicate that HHCB acts as a weak antagonist and agonist for the AR. Specifically, HHCB acted as an antagonist for the AR in HEK 293 and PALM cell lines (Cavanagh et al., 2018; Simmons et al., 2010; Schreurs et al., 2005; Schreurs et al., 2002). Anti-androgenic activity of HHCB was shown to be weaker than the positive control AR antagonists flutamide and vinclozolin (Schreurs et al., 2005) and bicalutamide (Cavanagh et al., 2018). HHCB showed no AR agonist activity in U2-OS and PALM cell lines transfected with human AR gene reporters (Cavanagh et al., 2018; Schreurs et al., 2005); however, it did show AR agonist activity in HEK 293 cells transfected with an AR gene reporter, although its transcriptional activity that was substantially weaker than that of testosterone (Li et al., 2023).

An OECD 443 EOGRT study found delayed preputial separation (by 1.4 days) in F1 males that did not show a monotonic response but was determined to be adverse in the highest dose group (a maternal dose of 91.7 mg/kg-day and a direct dose of 123.2 mg/kg-day starting at weaning) given concurrent decreases in bodyweight (IFF, 2021). The study also found decreased AGD in F2 males (16%), but not in F1 males, that was determined to be adverse at 45.6 mg/kg-day (achieved F0 maternal dose during gestation). The true adversity each of these findings is uncertain given that they occurred in different generations of offspring and given the suite of other hallmarks of anti-androgenicity that were unaffected (sperm parameters, nipple retention, histopathology in the male reproductive tract, etc.). Neither AGD nor age of preputial separation was measured in the other laboratory animal studies for HHCB; therefore, these endpoints cannot be evaluated for consistency across studies. However, a 90-day repeated dose oral toxicity study found no changes in weight or histopathology in androgen-sensitive tissues (prostate, seminal vesicles, and testes) in males at dietary exposure levels up to 156 mg/kg-day (Api and Ford, 1999).

A non-guideline study found an anti-androgenic pattern of effect in sexually mature male rats after intraperitoneal injection of a single dose of HHCB (20 mg/kg-day) for 30 days (Li and Wang, 2023). This included increased serum testosterone, corresponding decreases in LH and FSH, decreased sperm concentration and motility, increased abnormal sperm rate, and qualitative histopathology in the testes (convoluted seminiferous tubules were loosely packed, the intertubular gap was increased, and large vacuoles appeared in some convoluted seminiferous tubules). Notably, these endpoints were only measured in a single dose group (20 mg/kg-day), making it uncertain whether the observed effects were treatment-related vs. spurious. Also, several additional hallmarks of anti-androgenicity were unchanged: testes weight was not significantly altered, and no quantitative histopathological changes occurred in the testes (diameter of the seminiferous tubules and height of the epithelium). Furthermore, these findings were not replicated in guideline studies involving oral exposure to higher doses, which calls their

validity into question. Specifically, reproductive organ weights, histopathology, and sperm parameters were unaltered at doses up to 94.1 and 123.2 mg/kg-day, respectively, in F0 and F1 adult males in an EOGRT study and at doses up to 156 mg/kg-day in a 90-day repeated dose oral toxicity study (IFF, 2021; Api and Ford, 1999).

In contrast to the two studies above, an additional non-guideline study reported an androgenic pattern of effect in prepubertal male rats after intraperitoneal injection of a single dose of HHCB (20 mg/kg-day) for 7 days (Li et al., 2023). This included statistically significant increases in weights of androgen-dependent accessory sex tissues (67% in SV and 20% in prostate relative to control), increased proliferation of the SV and prostate epithelium, increased serum testosterone levels, and corresponding decreases in FSH and LH. Histopathological changes (increased folding of epithelial cells) occurred in the prostate, but not in the SV. No effects were found on testes weight or histopathology. For each of these outcomes, HHCB was less potent than an equivalent dose of exogenous testosterone, and most endpoints were only measured in a single dose group (20 mg/kg-day) making it uncertain whether the observed effects were treatment-related vs. spurious. Additionally, these findings were not replicated in guideline studies involving oral exposure to higher doses, which calls their validity into question. Specifically, relative weights and macroscopic observations were unaffected for the SV, prostate, or testes in an OECD 443 EOGRT study at doses up to 94.1 and 123.2 mg/kg-day, respectively in F0 and F1 adult males and in a 90-day repeated dose oral toxicity study at doses up to 156 mg/kg-day (IFF, 2021; Api and Ford, 1999). Furthermore, additional hallmarks of androgenicity (nipple regression, increased AGD, and accelerated preputial separation) did not occur in F1 males at doses as high as 123.2 mg/kg-day or in developmentally exposed F2 males in the EOGRT study.

In summary, *in vitro* studies suggest that HHCB is a weak antagonist, but not an agonist, for the AR. *In vivo* studies suggest that this potential activity is not potent enough to cause adverse effects on androgen-sensitive male reproductive endpoints at relevant exposures. Specifically, while HHCB caused anti-androgenic and androgenic effects in mature and pre-pubertal male rats, respectively, at a single dose in intraperitoneal injection studies, OECD 443 EOGRT and OECD 408 90-day oral toxicity studies generally showed no effects on the majority of relevant endpoints at higher doses and longer durations of exposure. Therefore, EPA is not further considering androgen disruption for dose-response analysis or for use in estimating risk to human health.

Estrogen Disruption: The available *in vitro* studies show slight evidence that HHCB has anti-estrogenic and selective estrogenic effects. Specifically, although HHCB demonstrated weak activity as an ER antagonist and a selective ER agonist in multiple cell lines, activity was less potent than that of known positive control antagonists and agonists. However, *in vivo*, HHCB showed no effects on estrogen-sensitive endpoints across a uterotrophic assay performed on juvenile Balb/c mice (Seinen et al., 1999), a 90-day repeated dose oral toxicity study performed in rats (Api and Ford, 1999), and an EOGRT study in rats (IFF, 2021). This is discussed below.

Available *in vitro* studies indicate that HHCB acts as an antagonist, but not an agonist, for the ER. Specifically, HHCB acted as an antagonist for ER alpha in yeast and in human HEK 293, and U2-OS cells and for ER beta in HEK 293 and U2-OS cells (Cavanagh et al., 2018; Simmons et al., 2010; Schreurs et al., 2005; Schreurs et al., 2002). Notably, the anti-estrogenic activity of HHCB was shown to be weaker than the positive control ER antagonists SERM 4-hydroxytamoxifen, ICI 182,780, and 4-OH-tamoxifen (Schreurs et al., 2005; Schreurs et al., 2002). HHCB showed weak (*i.e.,* orders of magnitude lower than estradiol (E2)) or no ER alpha agonist activity and no ER beta agonist activity in yeast or in human cell lines transfected with human ER gene reporters (HEK 293, U2-OS, HeLa, and MCF-7)

(Cavanagh et al., 2018; Simmons et al., 2010; Gomez et al., 2005; Schreurs et al., 2005; Schreurs et al., 2002; Seinen et al., 1999).

In vivo, three guideline studies show no evidence that oral exposure to HHCB alters estrogen-sensitive endpoints. Specifically, a uterotrophic assay in juvenile Balb/c mice found no effect of HHCB on uterus weight or thymus weight at dietary exposure levels as high as 40 mg/kg-day for 2 weeks (Seinen et al., 1999). Consistently, a 90-day repeated dose oral toxicity study performed in rats (Api and Ford, 1999), reported no evidence of hormonal effects after histopathological examination of the mammary gland, uterus and vagina of females at dietary exposure levels of up to 150 mg/kg-day. Furthermore, in an EOGRT study in rats, no effects on age of vaginal opening, estrous cycle parameters, fertility and reproductive performance parameters, or abnormal histopathological findings or weight changes in estrogen-sensitive tissues were noted in females of the F0 or F1 generations at dietary doses as high as 116 and 121 mg/kg-day (IFF, 2021).

In summary, *in vitro* studies suggest that HHCB has weak activity as both an agonist and an antagonist for the ER. *In vivo* studies suggest that this potential activity is not potent enough to cause adverse effects on estrogen-sensitive female reproductive endpoints. Therefore, EPA is not further considering estrogen disruption for dose-response analysis or for use in estimating risk to human health.

Maternal Toxicity and Associated Effects

Studies in laboratory animals provide moderate evidence that HHCB causes maternal toxicity that is not adverse until relatively high doses. This is supported by two developmental toxicity studies in rats and rabbits (IFF, Date Unknown-b; Christian et al., 1999; Argus Research Labs, 1997) and one OECD 443 EOGRT study in rats (IFF, 2021). Additionally, one of these studies provides slight evidence of skeletal malformations and decreased ossification after developmental exposures to HHCB; however, these did not occur until maternally toxic doses. This is discussed below.

Two prenatal developmental toxicity studies and an EOGRT reported effects on dam body weight, food consumption, and/or clinical signs at relatively high doses (>91.7 mg/kg-day in rats and 100 mg/kg-day in rabbits). Specifically, at the highest dose (91.7 mg/kg-day), an OECD 443 EOGRT study found slight, non-adverse decreases in F0 dam body weight (5–6% depending on the timepoint) and body weight gain (9% on GD 0–20), no changes in food consumption, and no clinical signs during gestation and even through lactation (IFF, 2021). Similarly, a prenatal developmental toxicity study in rats noted dose-dependent decreases in body weight gain and food consumption (percentages not reported and data not provided) in dams during GD 7 to 18 that did not occur until 150 mg/kg-day. Clinical signs were not observed until 500 mg/kg-day (Christian et al., 1999; Argus Research Labs, 1997). Furthermore, an OECD 414 study in rabbits found decreased body weight gain (11%) and food consumption (5%), but no signs of clinical toxicity in dams during GD 6 to 29 at the highest dose (100 mg/kg-day) (IFF, Date Unknown-b).

A non-guideline rat prenatal developmental toxicity study reported increased incidence of fetal skeletal malformations and decreased ossification of sternal centra and metatarsals in three fetuses from separate litters after gestational exposure to HHCB; however, these effects occurred at high, maternally toxic doses (*i.e.,* 500 mg/kg-day) (Christian et al., 1999; Argus Research Labs, 1997). An additional OECD 414 prenatal developmental toxicity study in rabbits (IFF, Date Unknown-b) reported no effects on skeletal malformations or ossification at the highest dose tested (100 mg/kg-day), which further suggests that this endpoint is either spurious, dependent on maternal toxicity, and/or does not occur until doses at or above 500 mg/kg-day.

Taken together, maternal toxicity and associated developmental effects are supported by a relatively small number of studies and are less sensitive than other reproductive endpoints from oral studies. Therefore, EPA is not considering maternal toxicity for dose-response analysis or for use in estimating risk to human health.

Effects After Gestational and Lactational Exposures: Decreased Offspring Body Weight

Because HHCB has been detected in human cord blood and breastmilk (see Section 2.2.2), EPA integrated evidence across all available studies involving gestational and lactational exposure to HHCB to determine the most suitable endpoint that protects for these PESS-related exposures. These studies include two prenatal developmental toxicity studies in and rabbits (IFF, Date Unknown-b) and rats (Christian et al., 1999; Argus Research Labs, 1997), and one OECD 443 EOGRT study in rats (IFF, 2021). These studies provide robust evidence that maternal exposure to HHCB causes decreased offspring bodyweight. These changes are dose-dependent, consistent, and are protective of PESS. This is discussed in more detail below.

Both the OECD 443 EOGRT study (IFF, 2021) and the associated range-finding study (IFF, 2020a) in rats show consistent effects on reduced offspring body weight that are biologically adverse, dose-dependent, and persistent across time in both sexes. Specifically, in F1 males in the EOGRT study, body weights decreased by 6 and 9% compared to control in the middle and high doses, respectively, on PND 1. By PND 21, F1 male body weights were decreased by 6 and 11% in the middle and high doses, respectively. By post-weaning day 120, F1 male body weights were decreased by 11 and 17%, respectively. In F1 females, body weights decreased 8% at the high dose on PND 1. By PND 21, F1 female body weights decreased by 5 and 11% in the middle and high dose, respectively. By lactation day 21, body weight remained decreased by 7% in the high dose group. Similar to the EOGRT study, in the range finding study, body weights in F1 males decreased by 8.7 and 11.7% relative to historical controls in the low and high dose groups respectively, on PND 1. By PND 21, F1 male body weights were decreased by 5.4 and 11.1% in the middle and high doses, respectively. Body weights in F1 females decreased by 8.8 and 9.9% relative to historical control in the low and high dose groups, respectively on PND 1. By PND 21, F1 female body weights were decreased by 3.8 and 9.2% in the low and high dose, respectively.

In addition to dose-dependence, consistency across time, and consistency across studies, decreases in offspring body weight persisted across generations in the EOGRT study. Specifically, in F2 males, body weights decreased significantly by 5% compared to control in the middle and high doses starting at PND 4. By PND 21, F2 male body weights were decreased by 7 and 12% in the middle and high doses, respectively. In F2 females, body weights decreased significantly by 5% compared to control in the high dose group starting at PND 7. By PND 21, F2 female body weights were decreased by 6 and 9% in the middle and high dose groups, respectively.

In prenatal developmental toxicity studies, effects on offspring body weight did not occur in rabbits at 100 mg/kg-day and did not occur in rats until maternally toxic doses of 500 mg/kg-day (IFF, Date Unknown-b; Christian et al., 1999; Argus Research Labs, 1997), it is important to note that, unlike the EOGRT study and the associated range finding study above, these studies measured fetal rather than pup body weight and involved exposure to HHCB for a portion of gestation-only. This suggests that effects of developmental exposure to HHCB on offspring body weights may depend on continuous exposure throughout the entire duration of gestation through parturition.

Additional findings in the EOGRT and prenatal developmental toxicity studies are limited, and this is discussed in more detail in the other evidence integration sections above. In each case, offspring

bodyweight changes in the EOGRT, which were adverse and statistically significant starting at a maternal dose of 45.6 mg/kg-day, are protective of these effects. In the EOGRT, other findings include dose-dependent decreases in AGD in F2 males (16 and 21% in the middle and high dose groups) and females (22 and 26% in the middle and high dose groups) starting at 45.6 mg/kg-day (achieved F0 maternal dose during gestation), and delayed preputial separation (by 1.4 days) in F1 males starting at 91.7 mg/kg-day (achieved F0 maternal dose during gestation). Notably, a lack of effects on additional androgen-sensitive endpoints reduced confidence in the adversity of these two findings. Reduced offspring bodyweights in the EOGRT are also protective of findings in the prenatal developmental toxicity study in rats. Specifically, this study found vertebral/rib malformations in three fetuses that were not litter mates after exposure to 500 mg/kg-day during GD 7 to 17 that was likely related to maternal toxicity (reduced food consumption, reduced maternal body gain during GD 7 to 10, excess salivation, urine-stained abdominal fur, red or brown substance on the forepaws, and alopecia) that occurred starting at 150 mg/kg-day (Christian et al., 1999; Argus Research Labs, 1997). Finally, the prenatal developmental toxicity study in rabbits did not identify any adverse effects in offspring. Therefore, as the most sensitive effect across these studies, reduced offspring bodyweight is sufficiently protective of PESS that are susceptible to exposures in cord blood and breastmilk.

In summary, the above studies suggest that HHCB has dose-dependent, biologically significant effects on offspring body weight that are consistent across sexes, over time, across generations, and across the two available studies that involved continuous exposure throughout gestation and into parturition. Furthermore, reduced bodyweight is protective of other effects in these studies and is therefore sufficiently protective of PESS that are susceptible to exposures in cord blood and breastmilk. Therefore, EPA is considering the outcome of decreased offspring body weight for dose-response analysis and for use in estimating risk to human health.

2.4 Non-Cancer Dose Response Assessment

2.4.1 Selection of Studies and Endpoints for Non-Cancer Dose-Response Analysis

EPA considered studies and non-cancer hazard endpoints from the suite of epidemiological and animal toxicology studies for which the weight of scientific evidence supported adverse health outcomes following HHCB exposure, as described in Section 2.2. This included one study and one hazard endpoint: Decreased offspring body weight reported in the F1 and F2 generations of the EOGRT study in rats (IFF, 2021). EPA reviewed whether this study and endpoint was relevant for each exposure duration when considering non-cancer PODs for estimating risks for acute, intermediate, and chronic exposure scenarios.

2.4.2 Non-Cancer Points of Departure (PODs) for Acute Exposures

Available oral, inhalation, and dermal studies indicate that HHCB is not acutely hazardous. Specifically, the available acute oral (OECD 423) (IFF, 2016b) and dermal (OECD 402) (IFF, 2016a) toxicity studies in rats both concluded that LD_{50}s exceeded 2,000 mg/kg-day. The available acute inhalation (OECD 403) (IFF, 2017) toxicity study in rats concluded that the 4-hour LC50 was greater than 5.04 mg/L. None of these studies noted any adverse clinical findings or changes in body weight.

Additionally, no health effects were observed across the available developmental toxicity studies that were relevant for setting an acute oral POD. Specifically, developmental endpoints considered to be relevant to acute exposure durations (*e.g.,* structural abnormalities, embryo lethality, fertility parameters, sperm abnormalities) were unaltered at the highest dose of 91.7/ 95.2 and 116.3/121 mg/kg-day in F1/F2 rats during gestation and lactation, respectively, in an OECD 443-compliant EOGRT study (IFF, 2021).

Similarly, no effects were found on the incidence of skeletal malformations at the highest dose of 100 mg/kg-day in a prenatal developmental toxicity study in rabbits (IFF, Date Unknown-b). Although a prenatal developmental toxicity study in rats identified vertebral/rib malformations in three fetuses that were not litter mates after exposure to 500 mg/kg-day during GD 7 to 17 (Christian et al., 1999; Argus Research Labs, 1997), these findings were likely related to maternal toxicity (reduced food consumption, reduced maternal body gain during GD 7 to 10, excess salivation, urine-stained abdominal fur, red or brown substance on the forepaws and alopecia) that occurred starting at 150 mg/kg-day.

In considering all reasonably available information, EPA has determined that it is unlikely any adverse effects will result following a single exposure to HHCB at concentrations relevant to human exposures. Therefore, the Agency has decided not to propose an acute non-cancer hazard value.

2.4.3 Non-Cancer Points of Departure (PODs) for Intermediate and Chronic Exposures

As stated above, EPA considered decreased F1 and F2 offspring body weight reported in the OECD 443 EOGRT study in rats to be appropriate for dose-response analysis and POD derivation for intermediate and chronic durations (IFF, 2021). This study was previously discussed in Section 2.3.3.2, as well as in the attached DER (U.S. EPA, 2026b).

As discussed in Section 2.3.3.2.5, several studies reporting developmental and reproductive effects due to HHCB exposure were inappropriate for dose-response due to study limitations and/or inconsistent findings across studies. First, collective findings from two non-guideline studies in male rats exposed via intraperitoneal injection support a LOAEL of 20 mg/kg-day (no NOAEL identified) based on histopathology in the seminal vesicle, testes, and/or prostate of immature and mature rats exposed for 7 days and 30 days, respectively, and based on sperm abnormalities in mature rats exposed for 30 days (Li and Wang, 2023; Li et al., 2023). Both studies tested a single dose for the majority of these endpoints, and furthermore, these findings were not replicated at dietary doses as high as 123.2 mg/kg-day in the F0 or F1 adult males of an OECD 443-compliant EOGRT study (IFF, 2021) or at dietary doses as high as 150 mg/kg-day in an OECD 408-compliant 90-day repeated dose oral toxicity study (Api and Ford, 1999). Therefore, it is uncertain whether these effects were incidental or treatment related.

Additionally, two prenatal developmental toxicity studies support NOAELs of 30 and 50 mg/kg-day (LOAELs of 100 and 150 mg/kg-day) based on decreased maternal body weight gain and food consumption in rabbits and rats, respectively (IFF, Date Unknown-b; Christian et al., 1999; Argus Research Labs, 1997). However, an OECD 443-compliant EOGRT study found no treatment-related effects on F0 dam body weight, food consumption, or clinical signs at similar doses (91.7 mg/kg-day during gestation) and longer durations of exposure (at least ten weeks prior to mating, during mating, during gestation, lactation, and up to the day prior to necropsy) (IFF, 2021). Finally, the range-finding study for the EOGRT study supported a LOAEL of 34 mg/kg-day (no NOAEL identified) based on decreased offspring body weight on PND 1 (8.7 and 8.8% in males and females, respectively) (IFF, 2020a). This study is not suitable for dose-response analysis because it did not include a concurrent control group and instead compared findings to historical control animals; however, it provides additional support for the effects of HHCB on offspring body weight in the extended one-generation study.

The OECD 443 EOGRT study in rats conducted by IFF (IFF, 2021) supports a NOAEL of 26.8 mg/kg-day and LOAEL of 45.6 mg/kg-day (during gestation) based on decreased body weights and body weight gains in F1 and F2 offspring. This NOAEL is protective of other effects noted in this study, including: dose-dependent decreases in AGD in F2 males (16 and 21% in the middle and high dose groups) and females (22 and 26% in the middle and high dose groups) starting at 45.6 mg/kg-day

(achieved F0 maternal dose during gestation), and delayed preputial separation (by 1.4 days) in F1 males starting at 91.7 mg/kg-day (achieved F0 maternal dose during gestation). This effect is further supported by an associated range-finding study, which observed decreased F1 offspring body weight on PND 1 (8.7 and 8.8% in males and females, respectively) at a similar LOAEL of 34 mg/kg-day (IFF, 2020a), although effects were compared to historical control data rather than concurrent study controls. Finally, this NOAEL is protective of other effects reported in the developmental/reproductive studies discussed above that were not suitable for dose-response.

Consistent with the 2012 Benchmark Dose Technical Guidance (U.S. EPA, 2012a), EPA conducted BMD modeling on body weight data from F1 male and female offspring combined to further refine the POD. EPA BMD modeled F1 body weight data at pre-weaning timepoints (PNDs 1, 4, 7, 14, and 21) because this period encompasses critical stages of development when decreased body weights are potentially indicative of other adverse developmental effects. EPA additionally modeled F1 body weight data at post-weaning timepoints. EPA did not BMD model F2 body weights because F1 and F2 body weights were equally sensitive at each respective timepoint, while achieved HHCB doses were lower for F1 pups; therefore, F1 body weight decreases are protective of F2 bodyweight decreases. EPA modeled all datasets using standard continuous models included in EPA's 2025 web-based version of BMD software (BMDS Online version 25.1) and considered BMRs of 5 and 10% relative deviation for pre-weaning and post-weaning timepoints, respectively. Details and results of BMD modeling of F1 offspring body weight are provided in *Draft Benchmark Dose Modeling Results for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (U.S. EPA, 2026a). Results from the best fitting model for each timepoint are also summarized below in Table 2-8. $BMDL_5$s ranged from 30 to 35 mg/kg-day in F1 offspring at pre-weaning timepoints and $BMDL_{10}$s ranged from 36 to 112 in F1 offspring post-weaning. EPA selected the lowest $BMDL_5$ of 30 mg/kg-day from all available timepoints in Table 2-8, which was from offspring body weights on PNDs 4 and 14, as the POD.

Table 2-8. Summary of BMD Modeling of Decreased F1 Rat Body Weight from (IFF, 2021) [a]

Dataset (Section with BMD Modeling Results)	Variance	BMR = 1 SD			BMR = 5% RD			BMR = 10% RD		
		Best Fitting Model	BMD_{1SD} (mg/kg-d)	$BMDL_{1SD}$ (mg/kg-d)	Best Fitting Model	BMD_5 (mg/kg-d)	$BMDL_5$ (mg/kg-d)	Best Fitting Model	BMD_{10} (mg/kg-d)	$BMDL_{10}$ (mg/kg-d)
F1 BW – PND 1	Constant	Exponential 3	133	78	Exponential 3	57	35	–	–	–
F1 BW – PND 4	Constant	Linear	144	84	Linear	51	30	–	–	–
F1 BW – PND 7	Constant	Exponential 3	116	71	Linear	48	31	–	–	–
F1 BW – PND 14	Constant	Exponential 3	61	45	Exponential 3	40	30	–	–	–
F1 BW – PND 21	Constant	Linear	56	43	Linear	42	33	–	–	–
Male F1 BW – At sexual maturation (≈PND 51)	Constant	Linear	142	97	–	–	–	Linear	107	75
Male F1 BW – Treatment day 50	Constant	Hill	52	31	–	–	–	Hill	57	36
Male F1 BW – Treatment day 72	Constant	Exponential 3	44	33	–	–	–	Exponential 3	48	38
Female F1 BW – At sexual maturation (≈PND 32)	Constant	Linear	150	101	–	–	–	Linear	145	99
Female F1 BW – Treatment day 50	Constant	Exponential 3	123	87	–	–	–	Exponential 3	156	112
Female F1 BW – Treatment day 72	Constant	Exponential 3	93	59	–	–	–	Exponential 3	108	71

'–' = BMR not evaluated for this dataset; BMD = benchmark dose; BMDL = 95% lower confidence limit of BMD; BMR = benchmark response; BW = body weight; PND = postnatal day; RD = relative deviation; SD = standard deviation

[a] This table presents the results for the best fitting model from each timepoint. For a comparison of fit across all models for each timepoint, refer to Draft Benchmark Dose Modeling Results for (HHCB) (U.S. EPA, 2026a).

EPA is proposing the $BMDL_5$ of 30 mg/kg-day based on decreased offspring body weight as the POD for assessing risks from intermediate and chronic durations of exposure. As discussed above, the $BMDL_5$ of 30 mg/kg-day is the best fitting model for bodyweights on PND 4 and 14 and is the lowest (most conservative) BMDL5 from among all of the available pre-weaning and post-weaning bodyweight datasets. This POD is protective of additional effects in the EOGRT study, as well as of maternal effects noted in other studies at higher doses (IFF, Date Unknown-b; Christian et al., 1999; Argus Research Labs, 1997). Additionally, the $BMDL_5$ is not constrained to one of the experimental doses within a given study, as a NOAEL or LOAEL would be, which may better define the POD (U.S. EPA, 2012a). The $BMDL_5$ was extrapolated to a human equivalent dose (HED) of 7.09 mg/kg-day using allometric body weight scaling to the three-quarters power (U.S. EPA, 2011c). A total uncertainty factor of 30 was selected for use as the benchmark margin of exposure based on an interspecies uncertainty factor (UF_A) of 3 (see Appendix D for further discussion) and an intraspecies uncertainty factor [UF_H] of 10).

2.4.4 Weight of Scientific Evidence

EPA concludes that the lowest HED of 7.09 ($BMDL_5$ of 30 mg/kg-day) supported by the OECD 443 EOGRT study in rats by IFF (IFF, 2021) is appropriate for calculation of risk from intermediate and chronic durations. A total UF of 30 was selected for use as the benchmark margin of exposure based on an interspecies (UF_A) of 3, and an intraspecies (UF_H) of 10. Consistent with EPA guidance (U.S. EPA, 2022, 2002b, 1993a), EPA reduced the UF_A from a value of 10 to 3 because allometric body weight scaling to the three-quarter power was used to adjust the POD to obtain a HED (Appendix A).

Selection of the EOGRT study and decreased offspring body weight as the key study and endpoint provides an important update to EPA's previous HHCB risk assessment, which noted uncertainty related to the lack of multigenerational reproductive toxicity studies on HHCB at the time (OCSPP, 2014). Furthermore, consideration of the EOGRT study allowed EPA to derive a POD that is based on an observed effect level ($BMDL_5$). Previously, EPA selected a NOAEL (20 mg/kg-day) that was the highest tested dose (Ford and Bottomley, 1997).

EPA has robust overall confidence in the selected POD based on the following weight of scientific evidence:

- ***Suitability for Dose-Response:*** EPA considered one hazard endpoint (decreased offspring body weight) and one study (an OECD 443-compliant EOGRT study in rats (IFF, 2021)) for dose-response and POD derivation. An additional study describing decreased body weight, although useful for weight of evidence, was not informative for dose response because it only tested two doses of HHCB and because it compared data to historical controls instead of concurrent study controls (IFF, 2020a). All other effects discussed in Section 2.3.3 and Appendix C (maternal toxicity, endocrine disruption, thyroid effects, and liver effects) were not considered for dose-response due to lack of biological significance/adversity, inconsistency across a limited number of studies, and/or evidence from a single study.
- ***Dose-Response and Temporal Concordance:*** In the EOGRT study (IFF, 2021), HHCB exposure resulted in treatment related decreases in offspring body weight. Decreased body weights were dose-dependent and biologically adverse (*i.e.,* ≥5 or 10% relative to concurrent controls in pre-weaning or post-weaning timepoints, respectively). Furthermore, body weight decreases persisted into adulthood in F1 males and females and until the last day of measurement (PND 21) in F2 males and females.
- ***Consistency Across Studies:*** Both studies that measured body weight after parturition in perinatally exposed animals support the NOAEL of 26.8 mg/kg-day and lowest LOAEL of 45.6

mg/kg-day identified in the EOGRT study (IFF, 2021). Specifically, Ford et al. (Ford and Bottomley, 1997) identified a NOAEL of 20 mg/kg-day at the highest tested dose, and IFF (IFF, 2020a) identified a LOAEL of 34 mg/kg-day at the lowest tested dose.

- ***Sensitivity:*** The $BMDL_5$ of 30 mg/kg-day based on reduced offspring body weight is protective of additional effects on delayed preputial separation in F1 males and decreased AGD in F2 males and females that occurred at NOAEL/ LOAELs of 45.6/91.7 and 26.8/45.6 mg/kg-day, respectively, in the same EOGRT study. It is also protective of maternal toxicity and associated effects in offspring from prenatal developmental toxicity studies (IFF, Date Unknown-b; Christian et al., 1999; Argus Research Labs, 1997).
- ***PESS Sensitivity:*** As the most sensitive developmental effect from an exposure window that included pre-mating through gestation, lactation, and sexual maturity, the proposed POD is expected to be adequately protective of sensitive lifestages, including pregnant women, infants, children, and adolescents. It is also protective of potential exposures to HHCB via cord blood and breastmilk, which are supported by biomonitoring data (see Section 2.2.2).

There are no studies conducted via the inhalation route that are relevant for extrapolating human health risk. Therefore, EPA is using the oral HED of 7.09 mg/kg-day to extrapolate to the inhalation route. The Agency assumes similar absorption for the oral and inhalation routes, and no adjustment was made when extrapolating to the inhalation route. For the inhalation route, EPA extrapolated the daily oral HEDs to inhalation HECs using a human body weight and breathing rate relevant to a continuous exposure of an individual at rest. Appendix A provides further information on extrapolation of inhalation HECs from oral HED. No dermal hazards are expected for HHCB (see Section 2.3.2.5); therefore, only oral and inhalation hazard values were derived.

2.5 Evaluation of the Carcinogenicity of HHCB Using the ReCAAP Weight-of-Evidence Framework

No cancer bioassays are available for HHCB. Hilton et al. (2022) published a weight of evidence-based framework for determining the need for rodent cancer bioassays for agrochemicals lacking chronic and/or carcinogenicity studies – known as the Rethinking Chronic Toxicity and Carcinogenicity Assessment for Agrochemicals Project, or the ReCAAP Framework. Although it was developed specifically for agrochemicals, EPA considers many of the same scientific principles in the ReCAAP Framework as applicable to TSCA risk evaluations. As such, elements of the ReCAAP Framework are used as an organizational tool to evaluate the extent to which the lack of carcinogenicity studies imparts significant uncertainty on the human health risk assessment for HHCB.

The ReCAAP framework takes into consideration multiple lines of evidence including information pertaining to nomenclature, physical and chemical properties; exposure and use patterns; ADME properties; and toxicological data (*e.g.,* genetic toxicity, acute toxicity, subchronic toxicity, hormone perturbation, immunotoxicity, and MOA). The framework was developed by a workgroup comprised of scientists from academia, government (including EPA), non-governmental organizations, and industry stakeholders. Recently, OECD has published several Integrated Approach to Testing and Assessment (IATA) case studies demonstrating applicability of the weight of evidence ReCAAP framework (OECD, 2024). Furthermore, EPA previously used the ReCAAP Framework to support the draft cancer human health hazard assessments for DIBP and DCHP, which received approval from the Scientific Advisory Committee in August 2025 (U.S. EPA, 2025).

Herein, EPA used some, but not all, elements of the ReCAAP framework and OECD case studies. Elements of the ReCAAP framework considered in this section include physical and chemical properties

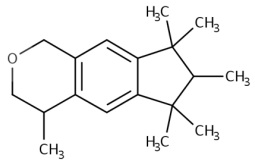
(Section 2.5.1), ADME properties (Section 2.5.2), acute toxicity (Section 2.5.3), subchronic toxicity (Section 2.5.4), genotoxicity (Section 2.5.5), evidence of hormone perturbation (Section 2.5.6), evidence of immune system perturbation (Section 2.5.7), cancer MOA (Section 2.5.8) and evidence of chronic toxicity and carcinogenicity from read-across to related chemicals (Section 2.5.8). This information is synthesized in a weight of evidence narrative provided in Section 2.5.10.

2.5.1 Physical and Chemical Properties

Table 2-9 presents physical and chemical properties for HHCB that are relevant to this ReCAAP assessment. For a more thorough characterization, refer to the *Draft Physical Chemistry, Fate and Transport, Environmental Release, and Environmental Exposure Assessment for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (U.S. EPA, 2026f). Values were obtained from the U.S. EPA's draft systematic review protocol for TSCA risk evaluations, furthermore,

- HHCB is a colorless viscous liquid at room temperature.
- HHCB has low vapor pressure.
- HHCB has low water solubility.
- HHCB has a log K_{ow} of 5.9 and is therefore lipophilic.
- HHCB may volatilize from water surfaces given the Henry's Law constant.

Table 2-9. Physical and Chemical Properties of HHCB

Property	Value
Structure	
Name (CASRN)	1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (1222-05-5)
Molecular formula	$C_{18}H_{26}O$
Molecular weight (g/mol)	258.41
Physical form	Viscous liquid
Melting point (°C)	≤ −20
Boiling point (°C)	325
Vapor pressure (mm Hg)	5.45E−04
Water solubility (mg/L)	1.75
Octanol/water partition coefficient (log K_{ow})	5.9
Henry's Law constant (atm·m^3/mol at 25 °C)	1.06E−04

2.5.2 Toxicokinetics

Data from human subjects and human epidermal membranes indicate that HHCB has low dermal absorption. Specifically, a study in three human volunteers detected less than 0.1% of the administered dose in urine, feces, and plasma, with 75% recovered at the site of application in dressing and skin washes (Ford et al., 1999). Consistently, a study in human epidermal membranes found that only 0.6% of the applied HHCB penetrated past the skin, with 81% recovered during washing (An-eX, 2001). As outlined in OECD guideline 428, this study calculated a conservative "total absorbable dose" by

measuring levels of HHCB *within* the skin and adding these to the amount that penetrated past the skin. This total absorbable dose, which likely over-estimates the true dermal absorption of HHCB, ranged from 5.2 to 8.85% depending on whether amounts from tape strips were included (see Section 2.2.1).

Additional experiments in rats and one pig indicate that HHCB is rapidly excreted (Api et al., 2013; Ford et al., 1999). Generally, levels of radioactivity in plasma, blood, and tissues reached peak concentrations within 5 minutes of intravenous injection and within 24 hours of dermal application, and then steadily declined through the last detection timepoint in each study. Specifically, in dermally exposed rats, 50% of the absorbed dose was eliminated between 24 and 48 hours, and 95% was eliminated at the study conclusion (120 hours) (Ford et al., 1999). Levels of radioactivity peaked at 6 hours for the liver and plasma; at 12 hours for the large intestine, small intestine, and stomach; and at 24 hours for the adipose tissue, before steadily decreasing to approximately 0.5 µg/g tissue in the large and small intestines and to less than 0.2 µg/g tissue in the stomach, liver, fat, and plasma at 120 hours. Similarly, in rats that received an intravenous injection of HHCB, 50% of the administered dose was eliminated by 24 hours; on day 7, 92% was eliminated (Api et al., 2013). Levels of radioactivity peaked at 5 minutes for the kidney, liver, whole blood, and plasma, and at 2 hours for the adipose tissue, before decreasing to below 0.1 µg/g in all measured tissues (adipose, kidney, liver, plasma, and whole blood). In a pig that received HHCB via intravenous injection, 50% of the administered dose was eliminated within 24 hours; by day 14, 88% was eliminated Api, 2013, 5427814. Radioactivity was measured in the skin and adipose tissue and declined (<0.3 ng/g skin and <3 ng/g adipose) at the conclusion (day 28).

HHCB was completely metabolized in rats and one pig; no unchanged compound was detected in urine of either species, and feces were not analyzed (Api et al., 2013). Although no studies have characterized the identities of these HHCB metabolites, no information is available to suggest that they are toxic.

Taken together, these human and rodent studies collectively indicate that HHCB has low dermal absorption. Any absorbed chemical is metabolized and rapidly excreted. Although HHCB has been detected in adipose tissue in human biomonitoring studies, concentrations in tissues, including adipose tissue, steadily decreased through the final timepoint in the available toxicokinetic studies, indicating low potential for bioaccumulation.

2.5.3 Acute Toxicity

The acute toxicity of HHCB has been evaluated by EPA and other regulatory agencies (NICNAS, 2019; OCSPP, 2014; ECB, 2008a, b). Consistent with previous assessments, EPA does not consider HHCB to be acutely toxic in terms of lethality via the oral, inhalation, or dermal route, and EPA does not consider HHCB to be an irritant or sensitizer. Table 2-10 summarizes the available acute oral and dermal LD50 values, inhalation LC50 values, as well as results from skin irritation, eye irritation, and skin sensitization testing (discussed in more detail in other sections of this draft TSD).

Table 2-10. Summary of Acute Toxicity Data for HHCB

Effect	Result
Oral LD_{50} (mg/kg)	>2,000 mg/kg-day (rat)
Dermal LD_{50} (mg/kg)	>2,000 mg/kg-day (rat)
Inhalation LC50 (mg/L)	> 5.04 mg/L (rat)
Skin Irritation	No effect
Eye Irritation	No effect
Skin Sensitization	Not a sensitizer

2.5.4 Subchronic Toxicity

No adverse effects on organ weights or histology were reported in an OECD 408 repeated dose 90-day oral toxicity study (Api and Ford, 1999) and in one 26-week subacute dermal toxicity study (IFF, Date Unknown-c) at doses as high as 150 and 130 mg/kg-day, respectively. Specifically, the oral study found no significant effects on organ weights (adrenals, brain, heart, kidneys, liver, lungs, ovaries, pituitary, spleen, testes, thymus and thyroids) or on histology in these tissues in addition to the aorta, brain, cecum, colon, duodenum, epididymides, eyes (including optic nerve), femur (including marrow), ileum, jejunum, lachrymal glands (exorbital), mainstem bronchi, mammary gland, mesenteric lymph node, esophagus, pancreas, prostate, rectum, salivary gland (submaxillary and sublingual), sciatic nerve, seminal vesicles, skin, spinal cord (three levels), sternum (including marrow), stomach, submandibular lymph node, trachea, urinary bladder, uterus and vagina (Api and Ford, 1999). The dermal study found no significant effects on organ weights (liver, kidneys, heart, brain, testes, ovaries, and uterus), or histopathological changes in the heart, kidneys, liver, brain, spinal cord, sciatic nerve, ovaries, uterus, testes, adrenals, eyes, large intestine, lymph nodes, pancreas, pituitary, urinary bladder, lungs, salivary gland, skin, small intestine, spleen, stomach, thyroid, traches, and esophagus) (IFF, Date Unknown-c).

As discussed in Appendix C.2 , exposure to HHCB was associated with increased relative liver weight in a 26-week subacute dermal toxicity study (NOAEL/LOAEL of 32.5/65 mg/kg-day), in F0, F1, and F2 generations of an EOGRT study (NOAEL/LOAEL of 26.8/45.6 mg/kg-day), in a range-finding study for the EOGRT (LOAEL of 34 mg/kg-day), and in a 2-week uterotrophic assay in mice (LOAEL of 6 mg/kg-day) (IFF, Date Unknown-c, 2021, 2020a; Seinen et al., 1999). Liver effects were considered to be non-adverse across these studies, because there were no concurrent pre-neoplastic lesions (*e.g.,* hepatocellular hypertrophy, cytomegaly, and hepatocyte necrosis) or serum chemistry markers of liver toxicity (*e.g.,* ALT, AST, ALP, GGT, bilirubin, cholesterol) that would potentially precede tumor formation.

2.5.5 Genotoxicity

Available studies evaluating the genotoxicity of HHCB include one micronucleus test in mice (Api and San, 1999) and several *in vitro* studies including a mouse lymphoma TK assay (IFF, 2020c), the Ames test (Api and San, 1999; Mersch-Sundermann et al., 1998a), a cytogenetics assay in Chinese hamster ovary (CHO) cells (Api and San, 1999), an unscheduled DNA synthesis (UDS) assay in primary rat hepatocytes (Api and San, 1999), a micronucleus test (MNT) with human lymphocytes (Kevekordes et al., 1997), a sister chromatid exchange test (Kevekordes et al., 1998), and an SOS chromotest (Mersch-Sundermann et al., 1998b). HHCB did not produce any genotoxic or mutagenic effects in these tests.

(Doğanlar et al., 2021) investigated the effect of long-term (*i.e.,* throughout 20 cell culture passage and exposure cycles over 60 days) exposure to HHCB at sub-lethal doses (5–2.5 µM) in U87 human glioblastoma cells and tumor spheroids. In U87 cells, HHCB treatment did not significantly change the number of migrated cells or wound closure capacity from transwell migration and wound healing assays, respectively. In tumor spheroids, HHCB did not significantly increase tumor invasion area in a Matrigel invasion assay. Using gel images from random amplified polymorphic DNA (RAPD) assays, the study authors reported a significant decrease to 74.5% “genomic template stability” in HHCB-treated tumor spheroids relative to control spheroids; however, this result has several associated limitations and uncertainties. Namely, because RAPD DNA fragmentation is a semi-quantitative method, it is possible that other factors such as differences in image saturation potentially confounded the results. Additionally, it is unclear how this endpoint was measured from the gels, as well as which gel (or lanes) corresponded to HHCB treated spheroids, because gels were not labeled. Finally, only a subset of the primers used in the experiment (6 of 14 total) were used to calculate genomic template stability. If all

primers were factored into the quantitative results, the differences between control and HHCB-treated spheroids would likely be minimal given the similarity of the banding patterns in the gel images. Given these uncertainties, results from this study generally indicate that long term exposure to HHCB did not produce genotoxicity *in vitro*.

Given that HHCB was negative for genotoxicity *in vivo* and in nine *in vitro* studies, EPA concluded that HHCB is not genotoxic. This conclusion is consistent with those of other regulatory organizations (NICNAS, 2019; OCSPP, 2014; ECB, 2008a, b).

2.5.6 Evidence of Hormone Perturbation

Androgen and Estrogen

The effects of HHCB on estrogen, androgen, and progesterone are discussed in detail in Sections 2.3.3.2.4 and 2.3.3.2.5.

Available *in vitro* studies indicate that HHCB acts as a weak antagonist and agonist for the human estrogen and androgen receptors (Cavanagh et al., 2018; Simmons et al., 2010; Gomez et al., 2005; Schreurs et al., 2005; Schreurs et al., 2002; Seinen et al., 1999) and for the human progesterone receptor (PR) in U2-OS cells (Schreurs et al., 2005). According to these studies, the activity of HHCB was much weaker than that of estradiol and testosterone, positive control ER antagonists ICI 182,780, tamoxifen, and 4-OH-tamoxifen; the positive control AR antagonists flutamide, vinclozolin, and bicalutamide; and the positive control PR antagonist RU486.

Available *in vivo* studies show no evidence of adverse, treatment-related effects of HHCB on androgen or estrogen-sensitive endpoints across three guideline studies involving the oral route of exposure. First, a uterotrophic assay in juvenile Balb/c mice found no effect of HHCB on uterus weight or thymus weight at dietary doses as high as 40 mg/kg-day for 2 weeks, confirming a lack of estrogenicity (Seinen et al., 1999). Additionally, an OECD 443 EOGRT study in rats found no effects on sperm parameters, relative weights and histopathology of androgen-sensitive tissues (testes, prostate, seminal vesicle, and epididymides), AGD in F1 offspring, nipple retention in F1 and F2 offspring, relative weights and histopathology of estrogen-sensitive tissues (ovaries, uterus, and mammary glands), estrous cycle parameters, fertility and reproductive performance parameters, and age of vaginal opening at dietary doses as high as 123.3 and 122.1 mg/kg-day in males and females, respectively (IFF, 2021). Third, in a 90-day repeated dose oral toxicity study in rats, histopathological examination of the prostate, seminal vesicles, and testes of males and of the mammary gland, uterus, and vagina of females did not reveal any evidence of hormonal effects at dietary exposure levels up to 150 mg/kg-day (Api and Ford, 1999).

Two non-guideline studies in prepubertal and mature male rats found that intraperitoneal injection of 20 mg/kg-day HHCB caused androgenic and anti-androgenic effects, respectively, in prepubertal and mature male rats. However, because three guideline studies that tested higher doses and relevant (*i.e.,* oral) exposure routes did not replicate these findings, EPA did not consider these two studies to be sufficient evidence to conclude that HHCB is an androgen agonist or antagonist.

Given that *in vivo* uterotrophic, OECD 443 EOGRT, and OECD 408 studies found no effects on androgen or estrogen sensitive endpoints, any agonist or antagonistic activity for the AR or ER shown *in vitro* is likely too weak to induce effects in humans at the current levels of exposure.

Thyroid

The effects of HHCB on thyroid signaling are discussed in detail in Appendix C.1. *In vitro*, HHCB was not an agonist or antagonist for the human thyroid hormone receptor in a Chinese hamster cell reporter

gene assay (Mori et al., 2007). *In vivo*, HHCB exposure increased thyroxine (T4), decreased thyroid stimulating hormone (TSH), increased thyroid weight, and/or induced thyroid histopathology in zebrafish, tadpoles, and in an EOGRT study in rats; however, none of these studies observed concurrent changes in multiple thyroid-related endpoints, making the individual findings questionable in terms of their biological significance. Specifically, one study in zebrafish larvae found significantly decreased T4 across all doses ranging from 0.13 to 3.10 ug/L, but did not investigate thyroid weights or histopathology (Chae et al., 2023), and an OECD 231 amphibian metamorphosis assay observed thyroid histopathology in tadpoles and frogs at the highest tested dose of 50 mg HHCB/kg food (Pablos et al., 2016) but did not measure T4 or TSH.

Although the hypothalamic-pituitary-thyroid axis and its associated cellular and molecular pathways are conserved between humans, zebrafish, tadpoles, and frogs, these studies should be interpreted with caution given that chemical agents are added to the tank water, which can lead to uncontrolled absorption that is not human-relevant and given that toxicokinetic properties like absorption and distribution are likely to differ across species. In the EOGRT study, relative thyroid weights increased dose-dependently and consistently across F0 and F1 adults; however, T4 and TSH changes were inconsistent across males and females for a given generation with no clear pattern of effect, and follicular cell hypertrophy occurred in the F0 generation, but not the F1 generation, despite the fact that the F1 generation was exposed to a similar dose for a longer period of time (post-weaning until PND 85-90) (IFF, 2021). Furthermore, no effects on thyroid weight or thyroid histopathology were detected at higher doses relative to the EOGRT study (150 mg/kg-day) in adult rats in an OECD 408 90-day oral toxicity study (Api and Ford, 1999). Finally, although chronic thyroid stimulation is a well-established mechanism that can lead to thyroid tumor formation in rats and mice, humans are less sensitive to this phenomenon because the plasma half-life of T4 in rodents is relatively short due to the considerable differences between species in the transport proteins for thyroid hormones (Capen, 1992). Therefore, effects noted in the extended one-generation study are of questionable human-relevance.

2.5.7 Evidence of Immune System Perturbation

An EOGRT study and a 90-day repeated dose oral toxicity study both reported no effects on immunotoxicity-related endpoints in rats at the highest tested doses of 123.3 and 150 mg/kg-day, respectively. Specifically, in the EOGRT study, no adverse, treatment-related effects were observed on immunophenotyping of splenocytes, weights or histopathology of lymphoid tissues (spleen, thymus, and lymph nodes), bone marrow cellularity, or on hematology parameters (leukocyte count, erythrocyte count, platelet count, and leukocyte differential count) in F0 and/or F1 adults at highest dietary doses of 123.3 and 122.1 mg/kg-day in males and females, respectively (IFF, 2021). Furthermore, in the 90-day repeated dose oral toxicity study, no adverse, treatment-related effects were reported for hematology parameters (total leukocyte count, platelet count, and leukocyte differential count) or weights and histopathology of lymphoid tissues at doses up to 150 mg/kg-day. Therefore, the available studies do not indicate that HHCB perturbs the immune system.

2.5.8 Mechanistic Studies to Support a Proposed Mode of Action

EPA did not identify any studies supporting a cancer MOA for HHCB. HHCB was found to be non-genotoxic in all of the available studies, and no studies are available that would support additional, non-genotoxic MOAs. Although HHCB exposure increased relative liver weight inconsistently in some repeat-dose oral exposure studies of rats and mice (see Appendix C.2), these studies found no concurrent pre-neoplastic lesions (*e.g.,* hepatocellular hypertrophy, cytomegaly, and hepatocyte necrosis) or serum chemistry markers of liver toxicity (*e.g.,* ALT, AST, ALP, GGT, bilirubin, cholesterol) that would potentially precede tumor formation.

2.5.9 Evidence of Chronic Toxicity and Carcinogenicity from Read-Across to Related Chemicals

No chronic toxicity or carcinogenicity studies are available for other polycyclic musks, such as acetyl hexamethyl tetralin (AHTN) and musk 89. EPA did not identify other chemicals that are suitable HHCB analogs for read-across from available studies and from previous assessments.

2.5.10 Weight of Scientific Evidence Conclusions Regarding Carcinogenicity of HHCB

Based on the weight of scientific evidence from the ReCAAP framework, EPA preliminarily concludes that the lack of chronic toxicity and carcinogenicity bioassays for HHCB do not suggest that there are significant remaining scientific uncertainties in the qualitative and quantitative risk characterization for this chemical. Further, EPA has preliminarily concluded that the non-cancer POD for HHCB based on decreased offspring body weight in rats from an EOGRT study that was selected for characterizing risk from intermediate and chronic exposure to HHCB is health-protective, including for potential cancer effects. These conclusions are based on the following weight of scientific evidence considerations:

- HHCB has low dermal absorption, with a lower bound of <0.1% based on a study in human subjects, and an upper bound of 5.2 to 8.1% based on a study in human skin samples. Any absorbed chemical is metabolized and rapidly excreted, with elimination rates ranging from 95% by day 5 in dermally exposed rats to 92% by day 7 in rats exposed via intravenous injection. Concentrations in blood and tissues peaked rapidly within 5 minutes of intravenous injection and within 24 hours of dermal application before steadily decreasing, suggesting a low potential for bioaccumulation despite its lipophilicity. (Section 2.5.2).
- EPA does not consider HHCB to be acutely toxic in terms of lethality via the oral, dermal, or inhalation route, and EPA does not consider HHCB to be an irritant or sensitizer (Section 2.5.3).
- Subchronic duration studies do not identify any additional target organs of HHCB toxicity based on a lack of organ weight changes and pre-neoplastic lesions (Section 2.5.4).
- HHCB is not considered to be genotoxic or mutagenic (Section 2.5.5).
- HHCB is a weak agonist and antagonist for the estrogen and androgen receptors, and a weak progesterone receptor antagonist *in vitro* in mammalian cells; however, available *in vivo* studies do not show evidence of effects on estrogen or androgen-sensitive outcomes in animals including an OECD 443 EOGRT study in rats (IFF, 2021), an OECD 408 repeated-dose 90-day oral toxicity study in rats that included histopathology for male and female reproductive organs (Api and Ford, 1999), and a uterotrophic assay in mice (Seinen et al., 1999). Additionally, HHCB was not an agonist or antagonist for the human thyroid hormone receptor *in vitro*; *In vivo*, thyroid effects were inconsistent across the OECD 443 EOGRT and OECD 408 studies and were likely related to increased clearance of T4 from liver enzyme induction, which is a rodent-specific mechanism (Section 2.5.6).

2.6 Consideration of PESS and Aggregate Exposure

2.6.1 Hazard Considerations for Aggregate Exposure

The health outcome (decreased offspring bodyweight) that serves as the basis for the intermediate/chronic hazard value for HHCB is systemic and assumed to be consistent across routes of exposure. EPA therefore concludes that for consideration of aggregate exposures, it is reasonable to assume that exposures and risks across oral, dermal, and inhalation routes may be additive for the selected POD in Section 2.7.

2.6.2 PESS Based on Greater Susceptibility

In this section, EPA addresses subpopulations likely to be more susceptible to HHCB exposure than other populations. Table 2-11 presents the data sources that were used in the PESS analysis evaluating susceptible subpopulations and identifies whether and how the subpopulation was addressed quantitatively in the draft risk evaluation of HHCB.

EPA examined sources of biological susceptibility for each of the susceptibility factors in the table below. The Agency quantitatively incorporated these considerations into hazard values and subsequent risk estimates when possible; however, for many factors EPA did not identify any reasonably available information to support quantitative adjustment of hazard/risk values. For these other factors, the Agency acknowledges either direct or indirect information suggesting additional susceptibility of certain subpopulations.

EPA was able to directly incorporate lifestage susceptibility into hazard values for the non-cancer endpoints. This is because the derived hazard value used for non-cancer risk characterizations is based on the most sensitive developmental effect (decreased offspring bodyweight) from an exposure window that included pre-mating through gestation, lactation, and sexual maturity; thus, it is protective of pregnant women, infants, children, and adolescents. It is also protective of potential exposures to HHCB via cord blood and breastmilk, which are supported by biomonitoring data (see Section 2.2.2). A UF_H factor of 10× was applied to account for human toxicokinetic and toxicodynamic variability, which is expected to account for considerations such as genetic polymorphisms and existing disease states.

Table 2-11. PESS Evidence Crosswalk for Biological Susceptibility Considerations

Susceptibility Factor	Examples of Specific Factors	Direct Evidence This Factor Modifies Susceptibility to HHCB		Indirect Evidence of Interaction with Target Organs or Biological Pathways Relevant to HHCB		Susceptibility Addressed in Risk Evaluation?
		Description of Interaction	Reference(s)	Description of Interaction	Reference(s)	
Lifestage	Embryos/ fetuses/infants Children	HHCB exposure (including gestation, lactation, and post-weaning in an EOGRT) resulted in decreased offspring body weight that persisted into adulthood	(IFF, 2021)			The most protective and best supported non-cancer POD is based on reduced offspring body weight.
	Pregnancy/ lactating status	HHCB exposure during gestation resulted in decreased weight gain and food consumption in dams	(Christian et al., 1999; Argus Research Labs, 1997); (IFF, Date Unknown-b)			Reduced maternal weight gain and food consumption is protected for by the reduced offspring body weight POD.
	Males of reproductive age	HHCB exposure (including gestation, lactation, and post-weaning in an EOGRT) potentially causes male reproductive effects including decreased AGD and delayed preputial separation (sexual maturation)	(IFF, 2021)			Decreased AGD and delayed preputial separation are protected for by the reduced offspring body weight POD.
	Elderly	No direct evidence identified				Use of default 10x UF_H
Pre-existing disease or disorder	Health outcome/ target organs	No direct evidence identified		Any pre-existing condition affecting a target organ will increase susceptibility to HHCB-toxicity in that organ. For example, several preexisting conditions may contribute to adverse developmental outcomes (*e.g.,* diabetes, high blood pressure, certain viruses).	(CDC, 2023c); (CDC, 2023d)	Use of default 10x UF_H
	Toxicokinetics	No direct evidence identified		Chronic liver disease is associated with impaired metabolism and clearance (altered expression of phase 1 and phase 2 enzymes, impaired clearance), which may enhance exposure duration and concentration of HHCB.		Use of default 10x UF_H

Susceptibility Factor	Examples of Specific Factors	Direct Evidence This Factor Modifies Susceptibility to HHCB		Indirect Evidence of Interaction with Target Organs or Biological Pathways Relevant to HHCB		Susceptibility Addressed in Risk Evaluation?
		Description of Interaction	Reference(s)	Description of Interaction	Reference(s)	
Lifestyle activities	Smoking	No direct evidence identified		Heavy smoking and other tobacco usage may increase susceptibility for reproductive outcomes.	CDC (2023a, 2023b)	Use of default 10x UF_H
Socio-demographics	Sex	Male rats demonstrated a more sensitive dose-response relationship for reduced body weight relative to females at post-weaning	(IFF, 2021)			Pre-weaning bodyweights had comparable sensitivity between males and females. Therefore, the POD is protective of ages where males and females had different sensitivity to reduced bodyweight. Use of default UF_H of 10
Genetics/ epigenetics	Health outcome/ target organs	No direct evidence identified		Polymorphisms in genes may increase susceptibility to developmental toxicity.		Use of default UF_H of 10
	Toxicokinetics	No direct evidence identified		Polymorphisms in genes encoding enzymes involved in metabolism of HHCB may influence bioaccumulation and clearance of HHCB.		Use of default UF_H of 10
Other chemical and nonchemical stressors	Chemical co-exposures	No direct evidence identified		HHCB is frequently mixed with solvents, fixatives, or other fragrance compounds, some of which could have developmental toxicity. For example, DEP, which commonly appears in mixtures with HHCB, has also been shown to cause developmental effects (including decreased postnatal body weight) in laboratory animals. Therefore, it is possible that DEP, or other chemicals, could compound the effects of HHCB, although there is no direct evidence.	(Weaver et al., 2020)	Qualitative discussion in text above and in this table

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2.7 Risk Assessment Approach and PODs Used to Estimate Non-Cancer Risks from HHCB Exposure

EPA concluded that no dermal hazards (see Section 2.3.2.5) or acute hazards (Section 2.4.2) are expected for HHCB at concentrations relevant to human exposure; therefore, only intermediate and chronic oral and inhalation hazard values were derived for use in the *Draft Risk Evaluation for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]2-benzopyran (HHCB)* (U.S. EPA, 2026i).

After considering hazard identification and evidence integration, dose-response evaluation, and weight of scientific evidence of POD candidates, EPA chose one non-cancer endpoint for use in determining the risk from intermediate and chronic exposure scenarios for the oral and inhalation routes (Table 2-12). The critical effect is reduced offspring body weight. EPA has robust overall confidence that the selected POD of 30 mg/kg-day (HED = 7.09 mg/kg-day) for intermediate and chronic durations is health protective. There are no studies conducted via the inhalation route relevant for evaluating human health risk. In the absence of inhalation studies, EPA performed route-to-route extrapolation to convert the oral HED to an inhalation human equivalent concentration (HEC) of 38.6 mg/m^3 (3.65 ppm). The HECs are based on daily continuous (24-hour) exposure, and HEDs are daily values.

The POD of 30 mg/kg-day (HED = 7.09 mg/kg-day; HEC = 38.6 mg/m^3 [3.65 ppm]) will be used in the *Draft Risk Evaluation for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]2-benzopyran (HHCB)* (U.S. EPA, 2026i) to estimate intermediate and chronic non-cancer risk from oral and inhalation exposures.

EPA is soliciting comments from the SACC and the public on the non-cancer hazard identification, dose-response and weight of evidence analyses, and the selected POD for use in risk characterization of HHCB. In particular, EPA is seeking SACC and public input on the following weight of evidence conclusions:

- HHCB is not acutely hazardous for any route of exposure and rationale to not derive hazard values or estimate risks for this exposure duration;
- HHCB is not hazardous via the dermal route and rationale to not derive hazard values or estimate risks for this exposure route; and
- the lack of chronic toxicity and carcinogenicity bioassays for HHCB does not suggest that there are significant remaining scientific uncertainties in the qualitative and quantitative risk characterization for this chemical.

Table 2-12. Non-Cancer Points of Departure Used to Estimate Risks to Human Health

Exposure Scenario	Target Organ System	Species	Duration of Exposure	POD (mg/kg-day)	Effect	HED [a] (mg/kg-day)	HEC [a] (mg/m^3) [ppm]	Benchmark MOE [b]	Reference	Overall Quality Determination
Intermediate, chronic	Developmental toxicity	Rat	Pre-mating in F0 generation through PND 21–23 in F2 generation	$BMDL_5$ = 30	Decreased body weight in F1 offspring on PNDs 4 and 14	7.09	38.6 [3.65]	UF_A=3 UF_H=10 *Total UF=30*	(IFF, 2021)	Acceptable/ Guideline [c]

BMDL = benchmark dose (lower 95th percentile); HEC = human equivalent concentration; HED = human equivalent dose; MOE = margin of exposure; POD = point of departure; UF = uncertainty factor

[a] HED and HEC values were calculated based on the most sensitive BMDL of 30 mg/kg-day.

[b] EPA used allometric body weight scaling to the ¾-power to derive the HED. Consistent with EPA Guidance (U.S. EPA, 2011c), the interspecies uncertainty factor (UF_A) was reduced from 10 to 3 to account remaining uncertainty associated with interspecies differences in toxicodynamics. EPA used a default intraspecies (UF_H) of 10 to account for variation in sensitivity within human populations.

[c] Reference was evaluated using the OPP DER format.

3 ENVIRONMENTAL HAZARD ASSESSMENT

3.1 Approach and Methodology

EPA completed searches for peer-reviewed and gray literature relevant references in September and May 2019, respectively. An update to the peer-reviewed literature search to capture information published since September 2019 was performed in May 2025 to identify any potential additional data sources for environmental hazard that might have been identified since the initial literature searches were conducted in 2019. EPA completed the review of environmental hazard data/information sources using the data quality review evaluation metrics and the rating criteria described in the 2021 *Draft Systematic Review Protocol Supporting TSCA Risk Evaluations for Chemical Substances* (U.S. EPA, 2021) and *Draft Risk Evaluation for HHCB – Systematic Review Protocol* (U.S. EPA, 2026j) and assigned an overall quality level of high, medium, low, or uninformative for dose response. EPA systematically evaluated all data for this hazard characterization but relied upon high-quality and medium-quality studies for quantitative hazard characterization. However, references receiving an overall quality determination of low or uninformative were included in tables and descriptions to provide complete summaries of the reasonably available information. Reasons for low or uninformative ratings included experimental doses exceeding the HHCB limit of water solubility, no effects at the highest concentration tested, and/or were part of a mixture of potentially hazardous chemicals.

HHCB may be distributed as a group of diastereomers and the properties may represent a combination of various substances that can result in variable physical and chemical properties (U.S. EPA, 2026f). The Agency does not have reasonably available information for environmental hazard endpoints on whether isomer mixtures or neat compounds were used.

EPA considered all available studies to characterize the environmental hazards of HHCB to ecological receptor groups, including aquatic vertebrates, aquatic invertebrates, algae, plants, terrestrial invertebrates, and terrestrial mammals. Mechanistic (gene expression) and behavioral endpoints were used to inform of the potential mechanisms that lead to the acute and chronic aquatic vertebrate hazard thresholds but were not included in calculations for hazard thresholds. Hazard studies with mammalian wildlife exposed to HHCB were not available; therefore, EPA used reproduction and development endpoints from human health laboratory rat and mouse model organisms to derive a toxicity reference value (TRV). The TRV was then used to extrapolate dietary thresholds from laboratory rodents to wild mammals while accounting for allometric scaling and differential feeding rates.

In aquatic species, EPA derived COCs to represent hazard thresholds. EPA uses probabilistic approaches (*e.g.,* SSD) when acute toxicity data from at least eight species are available (Raimondo and Barron, 2010) and uses deterministic approaches when data from at least eight species are not available. For HHCB, an SSD was derived for acute aquatic exposure hazards. Deterministic approaches were used to assess chronic hazard in aquatic vertebrates, sediment-dwelling animals, and terrestrial organisms.

An SSD is a model of the variation in sensitivity of species to a particular chemical stressor and is generated by fitting a statistical distribution to the proportion of species affected as a function of concentration or dose. This hazardous concentration (HC) is represented as an HCp, where p is the percent of species below the threshold. EPA used an HC05 (a hazardous concentration threshold for 5% of species) to estimate a concentration that is protective of 95% of species. The HC05 was then used to derive the COC as the lower bound of the 95th percentile confidence interval (CI) of the HC05 to account for uncertainty. EPA has more confidence in the probabilistic approach compared to the

deterministic approach when enough data are available because an HC05 is representative of a larger proportion of species in the environment. Empirical data included in the SSD analysis were limited to LC50 values (concentration lethal to 50% of test organisms) that were at or below the limit of water solubility of 1,750 µg/L for HHCB (U.S. EPA, 2020).

For the aquatic chronic vertebrate and sediment-dwelling animal deterministic approaches, COCs were derived after reviewing the lowest toxicity across a set of species for survival, reproduction, and growth endpoints. EPA calculated geometric means of the NOEC and LOEC values resulting in a chronic value (ChV) (U.S. EPA, 2002c). Assessment factors (AF) were used to extrapolate from the lowest or near lowest reasonably available hazard value to an environmental concentration that will be protective of most species in a medium. In this assessment, an AF of 10 was used to account for variation in sensitivity among species and uncertainties in extrapolating from laboratory conditions to environmental concentrations and durations (Zeeman, 1995; Zeeman and Gilford, 1993). For example, studies of chronic effects in laboratory conditions report hazards across concentrations, but at one standard exposure duration (*e.g.,* 7-day, 32-day, etc.). The AF accounts for environmental conditions where similar hazard effects may occur over longer exposure durations but at lower concentrations. Behavioral and mechanistic endpoints were considered as additional lines of evidence where appropriate (U.S. EPA, 2016).

For terrestrial species, EPA estimated hazard thresholds with the most sensitive fitness-related endpoints (*e.g.,* survival or reproduction) that ultimately lead to population-level adverse effects. For HHCB, a hazard threshold was calculated as the geometric mean of the lowest NOEC and LOEC (or NOAEL and LOAEL) combination for terrestrial mammals, terrestrial invertebrates, and terrestrial plants similar to the standard approach for aquatic organisms (U.S. EPA, 2002c; Zeeman, 1995). As a matter of policy, AFs are generally not applied by EPA when deriving hazard thresholds for terrestrial organisms. Chapman (1998) reviewed assessment factor extrapolations in terrestrial environments and concluded that there is no clear or consistent relationship between laboratory-based and field-based hazard effects on mammals and plants as being more or less conservative than the hazard concentration itself. Thus, AFs were not used to derive terrestrial hazard thresholds in this assessment.

3.1.1 Previous Environmental Hazard Assessments

Environmentally relevant assessments have been published by EPA (OCSPP, 2014) and the European Union (EU) (ECB, 2008b). Environment and Climate Change Canada (ECCC) has designated HHCB as a priority for assessment under the Canadian Environmental Protection Act, 1999 (CEPA) because of an information gathering initiative in 2017 (ECCC, 2017) and subsequent wastewater monitoring in Canada indicates that aquatic organisms may be exposed to HHCB through wastewater from both manufacturers and the use of products available to consumers (ECCC, 2024). HHCB is classified internationally as having acute and chronic hazards to aquatic organisms (ECB, 2008b; OCSPP, 2014). The EPA TSCA work plan assessment reported hazard COCs for acute exposure to aquatic animals (*Daphnia magna* 56.4 µg/L), chronic exposure to aquatic vertebrates (*Pimephales promelas* 9.7 µg/L), and chronic sediment exposure to sediment-dwelling animals (*Hyallela azteca* 1.08 mg/kg dry weight) (OCSPP, 2014). The EU risk assessment reported an overall Predicted No Effect Concentration (PNEC) to aquatic organisms of 4.4 µg/L based on the chronic exposure to the copepod *Acartia tonsa* to HHCB (ECB, 2008b). EPA considered these previous assessments as reasonably available information and uses them for context, comparison, and historical data. More studies on HHCB hazard, including hazards from soil exposures to terrestrial invertebrates and plants, have become available since these assessments were published and were considered reasonably available and evaluated in this assessment.

More recently, Gefell (2025) developed an extensive fish ecological hazard assessment of contaminants of emerging concern including HHCB in the Great Lakes-Upper St. Lawrence River drainage. Effect-specific ranges of screening values were developed from published adverse effect concentrations for HHCB behavioral, developmental, growth, mortality, reproductive and physiological/metabolic endpoints and compared to HHCB water concentrations measured across the Great Lakes Basin. Gefell (2025) reported the concentrations above which hazard to fish was relatively high for mortality (74.4 µg/L), reproduction (3.26 µg/L), behavior (35 µg/L), development (53 µg/L), growth (74 µg/L), and physiological/metabolic (2.84 µg/L).

3.1.2 Weight of Scientific Evidence

EPA uses several considerations when weighing the scientific evidence to determine confidence in the environmental hazard data. These considerations include the quality of the database, consistency, strength and precision, biological gradient/dose response, and relevance. This approach is described in 3.4.3 and the Draft Systematic Review Protocol (U.S. EPA, 2021).

3.2 Aquatic Species Hazard

EPA reviewed 35 studies for HHCB toxicity to aquatic organisms. Some studies included multiple endpoints, species, and test durations. Studies that received an overall quality determination of high or medium quality were used to derive hazard thresholds and are detailed in the subsections below. Studies that demonstrated no acute or chronic adverse effects at the highest concentration tested (unbounded NOECs), or where hazard values exceeded the limit of solubility for HHCB in water as determined by EPA at 1,750 µg/L, (OCSPP, 2014) are included in tables, but were excluded from consideration for the development of hazard thresholds (Section 3.4.2). One of the studies received an overall quality determination of low because it lacks information about the experiment, including the number of replicates per exposure group, the number of exposure groups, the concentration for all but the highest nominal concentration, and analytical verification of any exposure concentrations (Carlsson and Norrgren, 2004). This study is the only available study of HHCB hazard to embryos of aquatic animals and reports no developmental effects to fish embryos at acute exposures at the highest nominal concentration. These were not used to derive hazard thresholds for larval, juvenile, or adult animal endpoints. However, the study was reviewed and considered in the overall weight of evidence. Studies rated uninformative for dose response did not meet systematic review criteria. Five toxicity studies using spiked sediment for benthic exposures were identified for HHCB.

3.2.1 Acute Exposures to Aquatic Animals

EPA reviewed six high or medium quality studies and one low quality study for acute toxicity in aquatic vertebrates (Table 3-1). Six contained acceptable endpoints that identified definitive hazard values below the HHCB limit of water solubility (1,750 µg/L). One study of zebrafish embryos reports no hazard effects on developmental endpoints up to the nominal concentration of 1,000 µg/L HHCB over 48-hours (Carlsson and Norrgren, 2004). For tadpole larvae of the frog (*Rana nigromaculata*) and fish species, the 96-hour mortality LC50s ranged from 35.4 to 950 µg/L HHCB.

EPA reviewed eight high or medium quality studies and one low quality study for acute toxicity in aquatic invertebrates (Table 3-1). Seven contained acceptable endpoints that identified definitive hazard values below the HHCB limit of water solubility (1,750 µg/L) and reported mortality or immobilization endpoints after acute duration exposures. Hazard values ranged from 194 µg/L of 48-hour exposure to *Daphnia magna* to 861 µg/L to the larval midge *Chironomus plumosus*.

2321 **Table 3-1. Acute Aquatic Animal Toxicity of HHCB**

Test Organism	Hazard Values (µg/L)	Duration	Endpoint	Citation (Study Quality)
Vertebrates				
Frog (*Rana nigromaculata*)	35.4 (30–48) [a]	96-hour LC50	Larval mortality	(Fan et al., 2019) (High)
Chinese rice fish (*Oryzias latipes sinensis*)	839.2 (690–916) [a]	96-hour LC50	Larval mortality	(Fan et al., 2019) (High)
Pond loach (*Misgurnus anguillicaudatus*)	491.2 (464–512) [a]	96-hour LC50	Larval mortality	(Fan et al., 2019) (High)
Chinese rare minnow (*Gobiocypris rarus*)	753.8 (705–808) [a]	96-hour LC50	Larval mortality	(Fan et al., 2019) (High)
Japanese medaka (*Oryzias latipes*)	950 (910–1,010) [a]	96-hour LC50	Larval mortality	(Yamauchi et al., 2008) (High)
Zebrafish (*Danio rerio*)	>1,000 µg/L [b]	48-hour embryo development	Embryo	(Carlsson and Norrgren, 2004) (Low)
Zebrafish (*Danio rerio*)	4,450 [c] (3,460–5,690) [a]	96-hour LC50	Adult mortality [d]	(Zhang et al., 2012) (Medium)
	170/370 µg/L	120-hour NOEC/ LOEC	Adult oxidative stress biomarkers increased [e]	
Invertebrates				
Waterflea (*Daphnia magna*)	194 (137–253) [a]	48-hour LC50	Neonate immobilization	(Chen et al., 2015) (High)
	2,684 [c]	48-hour LC50	Neonate immobilization	(Fan et al., 2019) (High)
River prawn (*Macrobrachium nipponense*)	350 (326–382) [a]	96-hour LC50	Adult mortality	(Fan et al., 2019) (High)
Annelid *(Limnodrilus hoffmeisteri)*	2025 [c] (1,646–2,788) [a]	96-hour LC50	Mortality	(Fan et al., 2019) (High)
Annelid (*Lumbriculus variegatus*)	394 (221–706) [a]	120-hour LC50	Mortality	(Artola-Garicano et al., 2003) (High)
Midge *(Chironomus plumosus)*	861 (726–997) [a]	48-hour LC50	Larval mortality	(Fan et al., 2019) (High)
Midge (*Chironomus riparius*)	288 (105–351) [a]	96-hour LC50	Larval mortality	(Artola-Garicano et al., 2003) (High)
Copepod (*Acartia tonsa*)	470	48-hour LC50	Adult mortality	(Wollenberger et al., 2003) (High)
Copepod (*Nitocra spinipes*)	1,900 [c] (1,400–2,700) [a]	96-hour LC50	Adult mortality	(Breitholtz et al., 2003) (Medium)

Test Organism	Hazard Values (µg/L)	Duration	Endpoint	Citation (Study Quality)
Freshwater mussel (*Lampsilis cardium*)	999 (805–1,393) [a]	48-hour LC50	Larval mortality	(Gooding et al., 2006) (High)
	563 µg/L	96-hour EC50	Juvenile growth rate reduced 50%	(Gooding et al., 2006) (High)
Shrimp (*Palaemon varians*)	5/50 µg/L	24-hour NOEC/ LOEC	Behavior; 61% avoided HHCB compartment	(Ehiguese et al., 2019) (High)

EC50 = effect concentration at which 50% of test organisms exhibit an effect; NOEC/LOEC = no/lowest-observed-effect-concentration; LC50 = lethal concentration at which 50% of test organisms die
[a] 95% confidence interval around the LC50 as reported by the study authors.
[b] No effects up to and including the highest test dose.
[c] Measured concentration above HHCB limit of water solubility (1,750 µg/L).
[d] HHCB added to sediment, but water exposure concentrations measured and reported.
[e] Stress biomarkers include superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD).

3.2.1.1 Acute Animal COC

EPA developed an SSD for HHCB as there was empirical toxicity data for 12 species. The aquatic acute COC for HHCB was derived from an SSD that contained LC50s for one amphibian species, four fish species and seven invertebrate species identified in systematic review (Figure 3-1). SSDs were derived using EPA's SSD Toolbox (v1.1) (Etterson, 2020b) and plotted using R Statistical Software (v4.4.1) (R Core Team, 2019) using the ssdtools R package (v1.0.6) and the ggplot2 R package (v3.5.1; 3.4.3Appendix D). All studies included in the SSD were rated high or medium quality. The Maximum Likelihood method and a Weibull distribution model were used. The Weibull distribution was based on an examination of Akaike's Information Criterion corrected for sample size (AIC) for goodness of fit (Burnham and Anderson, 2002), visual examination of Q-Q plots, and evaluation of the line of best fit near the low-end of the SSD (Figure_Apx D-1). The HC05 for this distribution was 111.4 µg/L HHCB with a 95% confidence interval of 42.3 µg/L to 219.1 µg/L. After taking the lower 95th percentile confidence interval of this HC05 as an alternative to the use of assessment factors, the acute aquatic COC for vertebrates and invertebrates was 42.3 µg/L HHCB.

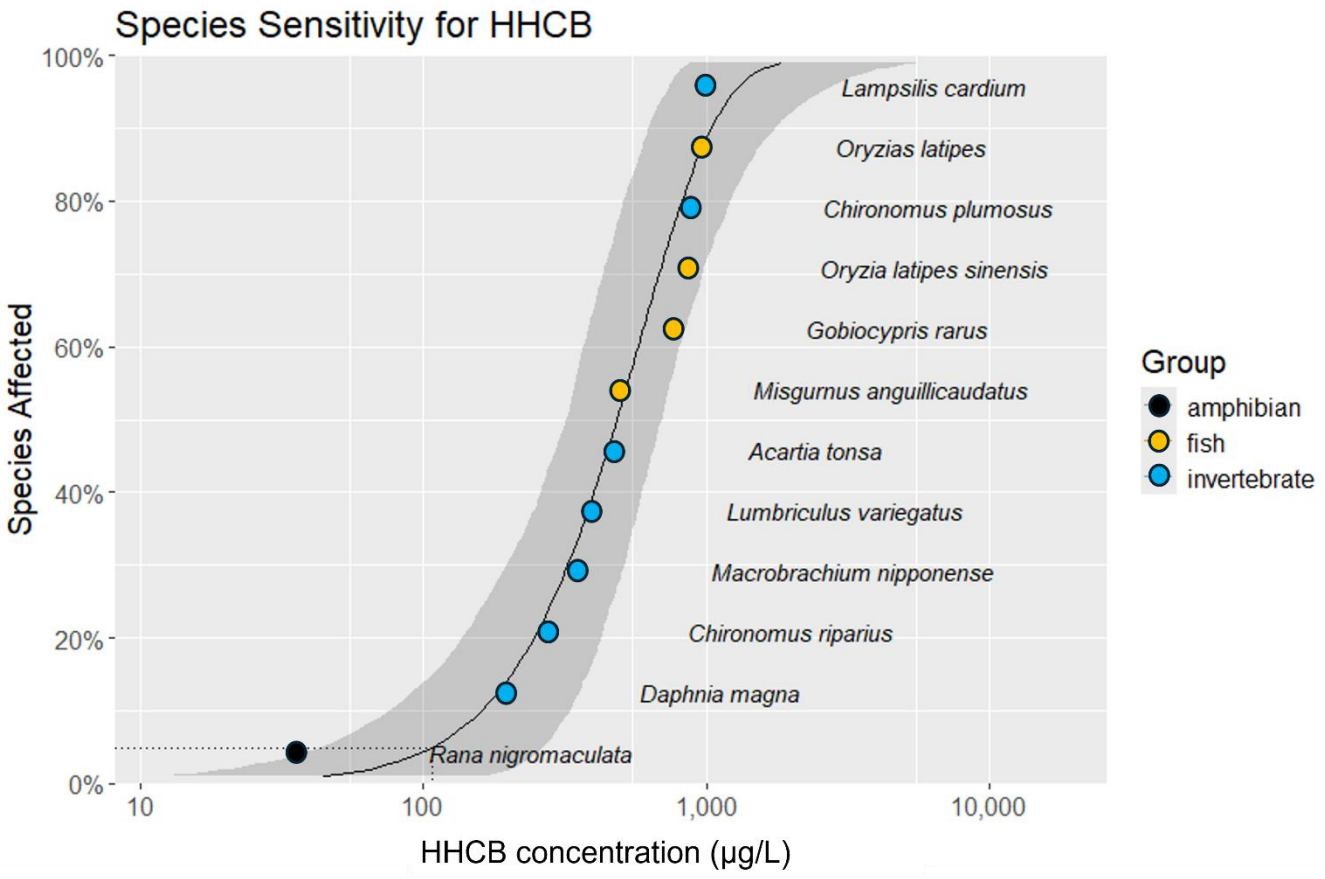

Figure 3-1. Species Sensitivity Distribution (SSD) of Acute Hazard Effects of HHCB on Aquatic Animals

The shaded band indicates the 95th percentile confidence interval. The dotted line indicates the 5% hazard concentration (HC05 = 111.4 µg/L).

The acute hazard distribution and subsequent COC is driven by one measured amphibian LC50. The measured LC50 of *Rana nigromaculata* (LC50 = 35 µg/L) was more sensitive to HHCB than the most sensitive measured LC50 for a fish species, *M. anguillicaudatus*, by an order of magnitude (LC50 = 491 µg/L), but was similar to EPA's hazard concentration that protects against mortality in 95th percentile of species (111.4 µg/L), and EPA's COC of 42.3 µg/L HHCB.

3.2.1.2 Weight of Scientific Evidence for Acute Animal COC

The weight of evidence suggests that HHCB poses acute hazard effects to vertebrate and invertebrate animals at 42.3 µg/L HHCB. EPA has robust confidence in this hazard threshold because the quality of the database of studies included 13 high- or medium-quality studies that consistently resulted in LC50s between 34.5 µg/L (Fan et al., 2019) and 839.2 µg/L HHCB (Fan et al., 2019). These studies all were conducted with reasonable dose-response designs and results, which enabled precise LC50 calculations (Table 3-1). These hazard effects were documented across a range of species and life stages that live in freshwater and marine environments in the water column as well as in or near the benthos/sediment. One study of zebrafish embryos reports no hazard effects on developmental endpoints up to 1,000 µg/L HHCB over 48-hours (Carlsson and Norrgren, 2004), suggesting minimal developmental hazard in fish prior to hatching.

EPA used a probabilistic technique (SSD) to derive a COC that is protective of 95th percentile of the aquatic animals in a community by incorporating hazard values across species and habitats. EPA has

robust confidence in the SSD with empirical data from 12 species because the species represent four different phyla (Annelida, Arthropoda, Chordata, and Mollusca) that inhabit both freshwater and marine environments. These data also include common test species (*e.g., Daphnia magna*) and less common amphibian and mussel species, thereby enhancing EPA's confidence that the COC is protective of a range of species. Limitations of an SSD include its reliance on model species that may not exist or interact in the same ecological community and are weighted equally. Also, the shape of the data distribution that is fitted to the effects data can be subjective and dependent on the three or four lowest values (Newman et al., 2000). Another assumption that may limit the scope of SSD inference is whether the number of species used is adequate. EPA used empirical data from 12 species in this HHCB assessment where the minimum number needed to fit a distribution is about eight species (Etterson, 2020b).

The use of Interspecies Correlation Estimation (ICE) models (*e.g.,* EPA Web-ICE) may be used to add surrogate LC50 data from additional species to SSD distributions where insufficient empirical data exist. However, adding modeled data also adds additional variation and epistemic uncertainty. Notwithstanding the limitations of SSD analyses, this method is widely used and accepted in risk assessments (Raimondo and Barron, 2010). The previous EPA TSCA work plan assessment did not use SSD probabilistic methods and arrived at an acute COC of 56.4 µg/L HHCB based on the most sensitive taxa, which was *D. magna*, and an assessment factor of 5 (OCSPP, 2014). Finally, this COC is within the same order of magnitude and more protective than the concentrations above which hazard to fish was relatively high for reproduction endpoints (74.4 µg/L) in the Great Lakes Basin (Gefell et al., 2025). EPA has robust confidence in the quality, consistency, strength and precision, and relevance of the studies used in determining the acute aquatic COC (42.3 µg/L HHCB).

3.2.2 Chronic Exposures to Aquatic Vertebrates

EPA reviewed four studies for chronic toxicity in aquatic vertebrates, three were high quality, and one was not rated because it studied the mechanistic endpoints of endocrine and neuron development (Table 3-2). All four studies contained acceptable chronic endpoints that identified definitive hazard values below the HHCB limit of water solubility (1,750 µg/L) for three fish species. Effects on fish body length and weight, behavior, and oxidative stress biomarkers occurred at 140 µg/L (Croudace et al., 1997), 182 µg/L (Wüthrich, 1996a), and 15 µg/L (Chen et al., 2012), respectively.

Croudace (1997) studied embryo and larval fathead minnow (*Pimephales promelas*) survival, standard body length, weight, and erratic behavior in 36-day (4-days pre-hatch and 32-days post-hatch) HHCB exposures in flow-through conditions. This study found no HHCB effects on embryo survival, but found 78% lower larval survival, 20% reductions in standard body length, 54% reductions in weight, and increased incidence of erratic swimming behavior in larval *P. promelas* in treatments with 140 µg/L HHCB (LOEC) (Croudace et al., 1997). No effects were observed at 68 µg/L HHCB (NOEC).

Wüthrich (1996a) reported the hazard effects of HHCB on juvenile bluegill (*Lepomis macrochirus*) growth in a 21-day flow through experiment. No effects of HHCB were observed at test concentrations up to 181.8 µg/L, but fish body length and weight were less than control treatments at 393 µg/L. Fish exhibited irregular respiration and bottom swimming in 181.8 µg/L (LOEC), but not in treatments with 92.5 µg/L (NOEC) HHCB.

Chen (2012) used a 21-day water exposure to goldfish (*Carassius auratus*) to study chronic effects of HHCB on fish oxidative stress biomarkers. This study found 25% increases in superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) activity in treatments with 15 µg/L HHCB (LOEC), but no difference from control treatments at 1.5 µg/L (NOEC) (Chen et al., 2012).

Chae (2023) exposed embryo-larval zebrafish to low concentrations of HHCB over 5 days. Thyroid hormone was reduced by at least 60% in all HHCB exposures including the lowest of 0.13 µg/L HHCB. Larval fish moved less in response to light changes at 0.96 µg/L HHCB compared to lower concentrations. Transcriptome analyses revealed changes in thyroid- and neuron-related genes at 0.13 µg/L and 0.96 µg/L HHCB respectively.

Table 3-2. Chronic Aquatic Vertebrate Toxicity of HHCB

Test Organism	Hazard Values	Duration	Endpoint	Citation (Study Quality)
Fathead minnow (*Pimephales promelas*)	**68/140 µg/L**	36-day NOEC/LOEC	Larval survival, growth, development, behavior	(Croudace et al., 1997) (High)
Bluegill (*Lepomis macrochirus*)	182/393 µg/L	21-day NOEC/LOEC	Juvenile weight reduced 47%, length reduced 12%	(Wüthrich, 1996a) (High)
	92/181.8 µg/L		Irregular respiration and bottom swimming	
	450 µg/L	21-day LC50	Juvenile mortality	
Goldfish (*Carassius auratus*)	1.5/15 µg/L	21-day NOEC/LOEC	Adult oxidative stress biomarkers increased 25% after 14-days [a]	(Chen et al., 2012) (High)
Zebrafish (*Danio rerio*)	0.13 µg/L	5-day LOEC	Thyroid development and regulation [b]	(Chae et al., 2023) (High)
	0.34/0.96 µg/L	5-day NOEC/LOEC	Neuron development and behavior [c]	

LC50 = Lethal concentration at which 50% of test organisms die; NOEC/LOEC = no/lowest-observed-effect concentration
[a] Stress biomarkers include superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD).
[b] Endocrine effects occurred at the lowest experimental concentration. Effects included 60% decrease in whole body thyroxine (T4), transcriptomic differences in thyroid regulatory genes, including downregulation of *ugt1ab* and *ttr* genes and upregulation of *crhβ* gene.
[c] Neuron development effects include upregulation of *c-fos* gene and downregulation of *mbp*, *gap43*, and *syn2a* genes. Behavior effects include decreased response distances to light stimuli.
Bolded value indicates the hazard effects used to derive the chronic HHCB COC for aquatic vertebrates.

3.2.2.1 Dietary Aquatic Vertebrates

EPA reviewed one high-quality study for toxicity of dietary exposure to the African clawed frog (*Xenopus laevis*) and found increased incidence of thyroid histopathology including hypertrophy, hyperplasia, and/or colloid in frog thyroid follicles when fed 50 mg/kg HHCB but found no effects on tadpole mortality, whole-body length, snout-to-vent length, or hind limb length ((Pablos et al., 2016); Table 3-3).

Table 3-3. Aquatic Vertebrate Toxicity of HHCB Via Dietary Exposure

Test Organism	Hazard Values	Duration	Endpoint	Citation (Study Quality)
African clawed frog (*Xenopus laevis*)	5/50 (mg/kg food)	23-day NOEC/LOEC	Tadpole accelerated development stage at day 14; increased thyroid follicles in frogs	(Pablos et al., 2016) (High)
	>50 (mg/kg food)	23-day NOEC/LOEC	No effect on tadpole mortality or size	
NOEC/LOEC = no/lowest-observed-effect-concentration				

3.2.2.2 Chronic Vertebrate COC

EPA reviewed eight high or medium quality studies for chronic toxicity in aquatic vertebrates (Table 3-2). The most sensitive organism with a clear population-level fitness endpoint was the fathead minnow (Croudace et al., 1997). This 32-day reproduction test of HHCB exposure to larval fish found 78% lower survival, 20% reduction in standard body length, 54% reduction in weight, and increased incidence of erratic swimming behavior in treatments with 140 µg/L HHCB (LOEC). No effects were observed at 68 µg/L HHCB (NOEC). Based on the presence of a clear dose-response relationship and a population-level fitness endpoint, the 32-day reduction in larval survival, length, and weight was selected to derive the chronic COC for aquatic vertebrates. EPA calculated the COC as the geometric mean of the LOEC (140 µg/L) and NOEC (68 µg/L), equal to 98 µg/L, and applied an AF of 10 resulting in a COC of 9.8 µg/L HHCB. An assessment factor of 10 was applied according to standard EPA methods and used to account for variation in species sensitivity and uncertainties in extrapolating from laboratory conditions to environmental concentrations and exposure durations (Section 3.1; (Zeeman, 1995; Zeeman and Gilford, 1993)).

3.2.2.3 Weight of Scientific Evidence for Chronic HHCB Exposures to Aquatic Vertebrates

The weight of evidence suggests that HHCB poses chronic hazard effects to vertebrate animals at 9.8 µg/L HHCB. EPA has robust confidence in the hazard threshold for six reasons. First, the reasonably available database of studies used for this determination includes three high quality studies to determine growth or reproduction effects using standard methods. Second, these studies were conducted on a range of different species including fathead minnow (*P. promelas*), bluegill (*L. macrochirus*) and goldfish (*C. auratus*) (Table 3-2). Third, these studies found consistent effects within the same order of magnitude of HHCB concentrations. Fourth, all of these studies were conducted with reasonable dose-response designs and results, which enabled precise estimations of effect concentrations. Finally, the 2014 TSCA EPA Work Plan Chemical Risk Assessment used the same study and arrived at the same COC (OCSPP, 2014). Thus, EPA has robust confidence in the quality, consistency, strength and precision, and relevance of the studies used in determining the chronic aquatic COC for vertebrates (9.8 µg/L HHCB).

3.2.3 Chronic Exposures to Aquatic Invertebrates

EPA reviewed four high-/medium-quality studies for chronic toxicity of HHCB in the water column to aquatic invertebrates (Table 3-4). Chronic exposure effects ranged from reduced reproduction in *D. magna* at 282 µg/L (Wüthrich, 1996b) to reduced larval development in marine copepod (*Nitocra spinipes*) at 20 µg/L (LOEC) (Breitholtz et al., 2003) over 3-week exposures. Effects on reproduction in another marine copepod species (*Acartia tonsa*) occurred at higher concentrations (131 µg/L EC50) over 6-day exposure durations (DHI, 2007). A COC for chronic exposure of HHCB to invertebrates was not

derived because the COC of chronic exposure to vertebrates is more sensitive and is thus protective of invertebrates.

Table 3-4. Chronic Aquatic Invertebrate Toxicity of HHCB

Test Organism	Hazard Values	Duration	Endpoint	Citation (Study Quality)
Waterflea (*Daphnia magna*)	293 µg/L	21-day EC50	Adult immobilization	(Wüthrich, 1996b) (High)
	282 µg/L	21-day EC50	Reproductive output reduced 50%	(Wüthrich, 1996b) (High)
Marine copepod (*Acartia tonsa*)	150/300 µg/L	6-day NOEC/LOEC	20% fewer eggs hatched, 2% body length reduction	(DHI, 2007) (Medium)
	131 µg/L	6-day EC50	Larval development rate reduced 50%	(DHI, 2007) (Medium)
Marine copepod (*Nitocra spinipes*)	10/20 µg/L	22-day NOEC/LOEC	Larval development reduced 30%	(Breitholtz et al., 2003) (Medium)
EC50 = effect concentration at which 50% of test organisms exhibit an effect; NOEC/LOEC = no/lowest-observed-effect-concentration				

3.2.4 Chronic Exposures to Sediment-Dwelling Animals

EPA reviewed five high quality studies for toxicity of HHCB in the sediment to sediment dwelling invertebrates including amphipods, midges, oligochaete worms, mud snails, and polychaete worms (Table 3-5). Chronic effects on sediment dwelling invertebrates ranged from reduced biomass after 28-day exposures to the freshwater amphipod *Hyalella azteca* in 16.3 mg/kg dw (IFF, 2004b) to lower egg production over 120-day exposures to a marine polychaete (*Capitella* sp.) in 123 mg/kg dw HHCB (Ramskov et al., 2009) in the sediment.

In three separate studies submitted by International Flavors and Fragrances with *H. azteca* (IFF, 2004b), *C. riparius* (IFF, 2004a), and *L. variegatus* (IFF, Date Unknown-a), nominal treatment concentrations are reported with the authors stating that the measured concentrations are reported elsewhere. The Agency did not have direct access to the measured concentration data, but both EPA (2014) and EU (2008b) report the measured concentrations. Thus, because of corroborating lines of evidence from these previous assessments, EPA used the measured concentrations reported in these previous assessments.

Hazards to *H. azteca* were determined in a 28-day sediment exposure experiment (IFF, 2004b). Measured concentrations were on average 49% of nominal. Total amphipod biomass per replicate was reported as 15% lower and body length was reported as 10% lower in treatments with measured sediment HHCB at 16.3 mg/kg dw. However, when calculated as individual biomasses and individual lengths, only length differed among amphipods. Amphipods were 11% shorter in sediment with measured concentrations of 16.3 mg/kg dw HHCB. No significant differences in individual biomass among treatments with surviving amphipods were found. No effects were observed at 7.1 mg/kg dw HHCB (NOEC). Egeler (2004b) also reported the concentration at which 50% lower biomass occurred (EC50) which was 53.5 mg/kg dw HHCB. This study followed standard OECD test guidelines for measuring effects of chemical exposure in sediment (OECD, 2010).

Hazards to the midge larvae (*C. riparius*) were also determined in a 28-day bioassay using first instar larvae with development rate and emergence ratio as hazard endpoints (IFF, 2004a). Measured concentrations are reported as 80% of nominal. Midge development rate was not affected up to the highest measured exposure concentration of 800 mg/kg dw HHCB. Midge emergence ratio differed in sediment with 400 mg/kg dw HHCB (LOEC) compared to control treatments and the NOEC treatment of 200 mg/kg dw. In this study, emergence ratio was defined as the sum of the midges emerged per experimental unit divided by the number of larvae introduced per experiment unit. Emergence ratio was 80% less in 400 mg/kg dw HHCB than in solvent controls.

Hazards to the blackworm, *L. variegatus*, were determined in a 28-day bioassay using adult worms with survival, reproduction, and biomass as endpoints (IFF, Date Unknown-a). Measured concentrations are reported as 60% of nominal. No differences in survival were observed across all HHCB sediment concentrations. Reproduction was 49% lower in treatments with 36.5 mg/kg dw measured HHCB (LOEC) than in solvent controls. The reported NOEC was 16.2 mg/kg dw measured HHCB. The EC50 for blackworm biomass was reported as 74.1 mg/kg dw measured HHCB.

Table 3-5. Sediment Dwelling Aquatic Invertebrate Toxicity of HHCB Through HHCB Additions to Sediment

Test Organism	Hazard Values [a] (mg/kg dw)	Duration	Endpoint	Citation (Study Quality)
Amphipod (*Hyalella azteca*)	7.1/16.3	28-day NOEC/LOEC	23% shorter body length	(IFF, 2004b) (High)
	53.5	28-day EC50	50% lower biomass	
Midge (*Chironomus riparius*)	200/400	28-day NOEC/LOEC	50% lower emergence ratio [b]	(IFF, 2004a) (High)
	>800	28-day EC50	No effect on development	
Annelid (*Lumbriculus variegatus*)	**16.2/36.5**	28-day NOEC/LOEC	49% lower reproduction	(IFF, Date Unknown-a) (High)
	74.1	28-day EC50	50% lower biomass	
New Zealand mud snail (*Potamopyrgus antipodarum*)	7/19.3	120-day NOEC/LOEC	Juvenile growth rate reduced 35%; feeding rate reduced 30%	(Pedersen et al., 2009) (High)
Polychaete (*Capitella* sp.)	26/123	120-day NOEC/LOEC	33% Fewer eggs produced	(Ramskov et al., 2009) (High)
	123/168	120-day NOEC/LOEC	15% greater juvenile mortality	

EC50 = effect concentration at which 50% of test organisms exhibit an effect; NOEC/LOEC = no/lowest-observed-effect-concentration
[a] hazard of HHCB in sediment measured in mg of HHCB per kg dry weight (dw) of sediment.
[b] emergence ratio was the sum of emerged midges per experimental vessel divided by the number initially introduced larvae per vessel.
Bolded value indicates the hazard effects used for deriving the HHCB hazard threshold for sediment-dwelling invertebrates.

3.2.4.1 Sediment-Dwelling Animal COC

EPA reviewed five high quality studies for sediment exposure hazard effects to sediment dwelling invertebrates (Table 3-5). The two most sensitive organisms for which reproduction or growth effects were observed were the freshwater blackworm *L. variegatus* (IFF, Date Unknown-a) with a population-level LOEC of 36.5 mg/kg dw HHCB and the amphipod *H. azteca* (IFF, 2004b) with an individual growth reduction LOEC of 16.3 mg/kg dw HHCB. EPA derived the COC using the population-level hazard effects demonstrated by sediment HHCB exposures to *L. variegatus*. This 28-day test of sediment exposure found worm reproduction, measured as the total number of worms (*i.e.,* population of worms) in experimental vessels to be 49% lower in treatments with measured sediment HHCB concentrations at 36.5 mg/kg dw (LOEC). No effects were observed at 16.2 mg/kg dw HHCB (NOEC). This study followed standard OECD test guidelines for measuring effects of chemical exposure in sediment (OECD, 2010) with a well-established test species that is widely distributed across the Unites States (Thorp and Covich, 2001).

Based on the presence of a clear dose-response relationship and a population-level fitness endpoint, the 28-day reduction in reproduction was selected to derive the COC for sediment dwelling invertebrates. EPA calculated the COC as the ChV (chronic value) derived from the geometric mean of the LOEC (36.5 mg/kg dw HHCB) and NOEC (16.2 mg/kg dw HHCB), equal to 24.3 mg/kg dw, and applied an AF of 10 resulting in a COC of 2.4 mg/kg dw HHCB. An AF of 10 was applied according to standard EPA methods and used to account for variation in species sensitivity and uncertainties in extrapolating from laboratory conditions to environmental concentrations and exposure durations (Section 3.1; (Zeeman, 1995; Zeeman and Gilford, 1993)).

3.2.4.2 Weight of Scientific Evidence for Chronic Sediment-Dwelling Animal COC

The weight of evidence suggests that HHCB poses hazard effects to sediment dwelling invertebrates at 2.4 mg/kg dw HHCB. EPA has robust confidence in the hazard threshold for a number of reasons. First, the reasonably available database of studies used for this determination includes five high quality studies to determine growth or reproduction effects using standard methods. Second, these studies were conducted on a range of different species including amphipods (*H. azteca*), midge larvae (*C. riparius*), annelids (*L. variegatus*), mud snails (*P. antipodarum*), and marine polychaetes (*Capitella* sp.) (Table 3-5). Third, these studies found consistent effects within the same order of magnitude of HHCB concentrations. Finally, all the studies were conducted with reasonable dose-response designs and results, which enabled precise estimations of effect concentrations. Therefore, EPA has robust confidence in the quality, consistency, strength and precision, and relevance of the studies used in determining the chronic aquatic COC for sediment dwelling invertebrates (2.4 mg/kg dw HHCB).

3.2.5 Algae

EPA reviewed one high-quality study for short-term (72-hour) toxicity of HHCB to algae populations and found 50% reduction in population growth of (*Pseudokirchneriella subcapitata*) at 720 µg/L HHCB over 72-hours (Van Dijk, 1997) (Table 3-6). A COC for chronic exposure of HHCB to algae was not derived because the COC of chronic exposure to vertebrates is more sensitive and is protective of algae.

Table 3-6. Algal Toxicity of HHCB

Test Organism	Hazard Values (µg/L)	Duration	Endpoint	Citation (Study Quality)
Green algae (*Pseudokirchneriella subcapitata*)	720	72-hour EC50	Population growth rate reduced 50%	(Van Dijk, 1997) (Medium)
	204/466	72-hour NOEC/LOEC	Reduced population growth rate	
EC50 = effect concentration at which 50% of test organisms have an effect; NOEC/LOEC = no/lowest-observed-effect-concentration				

3.3 Terrestrial Species Hazard

EPA identified 14 terrestrial toxicity studies as the most relevant for quantitative assessment.

3.3.1 Terrestrial Vertebrates

Hazard studies with mammalian wildlife exposed to HHCB were not available, therefore EPA used ecologically relevant endpoints from human health laboratory rat and mouse model organisms to establish a hazard threshold for terrestrial mammals. In lieu of wild mammal hazard data, EPA identified five laboratory rodent diet studies that represent terrestrial mammal hazards (Table 3-7). In an extended one-generation reproductive toxicity study (OECD Guideline 443 compliant) of dietary exposure, International Flavors and Fragrances (2021) established a NOAEL of 26.8 mg/kg bw/day and a LOAEL of 45.6 mg/kg bw/day based on a greater or equal to 5% decrease in F1 and F2 offspring bodyweight (statistically significant decreases at the LOAEL ranged from 5–6% from PND 1 to 21 in F1 offspring and from 5–7% in F2 offspring). Descriptions of these laboratory rodent studies can be found in Section 2.3.3.2. The geometric mean of this NOAEL and LOAEL is 40.0 mg/kg bw/day.

Table 3-7. Terrestrial Mammal Toxicity of HHCB via Oral Exposure to Laboratory Rodents

Test Organism	Hazard Values	Duration	Endpoint	Citation (Study Quality)
Norway rat (*Rattus norvegicus*) Wistar	**26.8/45.6 mg/kg-day**	Extended one-generation (10 weeks pre-mating F0 through PND21–23 of F2 pups)	5-6% lower pup weight in F1 pups and 5-7% lower pup weight in F2 pups (PND 1-21)	(IFF, 2021) (Acceptable/Guideline)
Norway rat (*Rattus norvegicus*) Wistar	38/134 mg/kg/day	71-day test (14-day parental diet exposure) diet Developmental NOAEL/LOAEL	12% lower pup weight	(IFF, 2020a) (High)

Test Organism	Hazard Values	Duration	Endpoint	Citation (Study Quality)
Norway rat (*Rattus norvegicus*) Sprague-Dawley	150/500 mg/kg/day	13-week gavage Developmental NOAEL/LOAEL	Axial skeleton malformation	(Christian et al., 1999) (High)
	50/150 mg/kg/day	13-week gavage Maternal NOAEL/LOAEL	Reduced weight gain and feed consumption	
	150/500 mg/kg/day	20-day gavage Developmental NOAEL/LOAEL	Reduced fetal body weight; Increased fetal malformation	(Argus Research Labs, 1997) (Low)
	50/150 mg/kg/day	20-day gavage maternal NOAEL/LOAEL	Reduction in maternal body weight	
	>150 mg/kg/day [a]	90-day oral feeding	Body weight; histopathology	(Api and Ford, 1999) (Medium)

NOAEL/LOAEL = no/lowest-observed-adverse-effect level
[a] no effects up to and including the highest test dose.
Bolded value indicates the hazard effects used for deriving the HHCB hazard threshold for terrestrial mammals (see Section 3.3.1).

3.3.1.1 Terrestrial Vertebrate Hazard Thresholds

Five laboratory rat studies were assessed with the most sensitive reproductive endpoint value chosen to represent the terrestrial mammalian hazard threshold (Table 3-7). Reproductive endpoints were chosen to represent the potential hazards that may scale-up from individual effects to population-level effects. The hazard threshold was derived from the most sensitive among the acceptable-quality studies involving the Wistar strain of Norway rat (*R. norvegicus*) (IFF, 2021), with an extended one generation LOAEL of 45.6 mg/kg-bw/day HHCB and NOAEL of 26.8 mg/kg-bw/day for reducing pup weight by up to 7%. EPA calculated a geometric mean of the NOAEL and LOAEL from this study to equal 35.0 mg/kg-bw/day HHCB. This rat hazard threshold of 35.0 mg/kg-bw/day HHCB was used to screen risk estimates of potential HHCB dietary exposure to wild mammals.

3.3.1.1.1 Hazard Thresholds for Wild Mammals

Terrestrial mammals are expected to be exposed to HHCB through their diet via an aquatic pathway of bioconcentration in fish and secondary poisoning to mammals that consume fish. An additional pathway of HHCB mammal exposure is through a land pathway from biosolid application to soil, earthworm ingestion of soil, and wild mammal ingestion of earthworms. EPA used the hazard threshold value of 35 mg/kg-bw/day HHCB from the laboratory-based rat study as both a screening hazard threshold and an input of mammalian HHCB toxicity into the KABAM Model (EPA's K_{OW} [based] Aquatic Bioaccumulation Model version 1.0) (U.S. EPA, 2009)) that was used to refine the estimates of the potential HHCB bioaccumulation in freshwater ecosystems and subsequent risk to mammals.

EPA used equations for allometric food intake relationships for wild mammals from the EPA *Wildlife Exposure Factors Handbook* (U.S. EPA, 1993b) and KABAM's internal food ingestion equations based on the body weights of different species (U.S. EPA, 2009). KABAM calculates the dietary-based threshold assuming that HHCB intake is a function of the HHCB in the food of the mammal while not

considering exposure through drinking water intake or the relative difference in food consumed per day of mammals of different sizes. Using a standard feeding rate to body weight ratio of 0.05 (*i.e.,* most mammals consume 5% of their body weight in dry food per day (U.S. EPA, 1993b)) results in a dietary-based hazard threshold that is species independent and 700 mg/kg-diet.

For evaluating different receptor species, EPA converted this dietary hazard threshold into species-specific and dose-based hazard thresholds using species-specific food intake rates. The dietary and adjusted dose-based hazard values using the body weights of mammals ranged from 13.68 mg/kg-bw HHCB for large river otters to 73.50 mg/kg-bw HHCB for small fog shrews and water shrews (Table 3-8).

Table 3-8. Representative Mammal Body Weights and Dose-Based Adjusted Hazard Values

Mammal Species	Body Weight (kg) [a]	Dietary-Based Hazard Value (mg/kg-diet)	Dosed-Based Hazard Value (mg/kg-bw)
Fog shrew (*Sorex sonomae*) Water shrew (*Sorex palustris*)	1.8E-02	700	74
Rice rat (*Oryzomys palustris*) Star-nosed mole (*Condylura cristata*)	8.5E-02	700	50
Small mink (*Mustela vison*)	0.45	700	33
Large mink (*Mustela vison*)	1.8	700	23
Small river otter (*Lontra canadensis*)	5.0	700	18
Large river otter (*Lontra canadensis*)	15	700	14
[a] High-end body weights were used for shrews and moles while low-end and high-end body weights for large and small mink and otter from U.S. EPA (1993b) and also used in KABAM (U.S. EPA, 2009).			

3.3.1.2 Weight of Scientific Evidence for Terrestrial Vertebrate Hazard Threshold

No studies on terrestrial wildlife involving mammals were identified. In lieu of terrestrial wildlife studies, five references for rat studies as human health model organisms were used to determine a lowest and most conservative HHCB concentration that affected apical endpoints (survival, reproduction, growth) in rodents and that could serve as a conservative upper bound of for wild mammal populations in a screening assessment.

The weight of evidence suggests that HHCB poses chronic dietary exposure hazard effects to terrestrial mammals at 35.0 mg/kg-bw/day HHCB. EPA has robust confidence in this hazard threshold for three reasons. First, the reasonably available database of studies used for this determination include one acceptable guideline, two high, one medium, and one low quality studies to determine reproductive effects of HHCB using standard methods. The terrestrial mammalian hazard threshold was derived from the most sensitive among acceptable-quality studies involving the Wistar strain of Norway rat (*R. norvegicus*) (IFF, 2021), with an extended on-generation LOAEL of 45.6 mg/kg-bw/day HHCB and NOAEL of 26.8 mg/kg-bw/day for 6 to 15% lower pup weight. Second, the three highest rated studies found consistent effects within the same order of magnitude of HHCB doses. Finally, all of the studies were conducted with reasonable dose-response designs and results, which enabled precise estimation of effect concentrations.

Population level effects were not observed in ecologically relevant species. Considerable uncertainties surround whether or how these effects on individual growth and reproductive development translate into effects on wild mammal fitness and population parameters. Because of these uncertainties of

extrapolations to wildlife mammal species, EPA has moderate confidence that the hazards are representative of the range of wild mammal species. Therefore, EPA has robust confidence in the quality, consistency, and strength and precision, of the studies used in determining the TRV 35.0 mg/kg-bw/day HHCB. The Agency then derived hazard thresholds to wild mammals using the TRV using conservative inputs of body weight, feeding rate, and assimilation efficiency. Because of these inputs, EPA has moderate confidence in hazard threshold values for the wild mammals list in Table 3-8.

3.3.2 Terrestrial Invertebrates

EPA reviewed six high-quality studies and one low-quality study for acute toxicity of HHCB to terrestrial invertebrates (Table 3-9). Mortality effects (LC50) from 14-day soil exposures to earthworms (*Eisenia fetida*) ranged from 188 mg/kg dw (Wang et al., 2015) to 392 mg/kg dw (Chen et al., 2011a) HHCB. Short-term filter paper HHCB additions (OECD No. 207) resulted in an LC50 value of 6.14 µg/cm^2 (Chen et al., 2011b). Hazard effects on earthworm reproduction occurred at soil HHCB concentrations from 50 mg/kg dw (Chen et al., 2011a) to 105 mg/kg dw (Goßmann, 1997). Chen (2011a) found 30% fewer cocoons produced with 50 mg/kg dw HHCB in soil compared to earthworms in soil with 30 mg/kg dw HHCB and in soil without HHCB. One study exposed the nematode to HHCB in water and reported mortality, growth, and reproduction hazards at concentrations above the HHCB limit of water solubility (1,750 µg/L; (U.S. EPA, 2020; Mori et al., 2006). Fifty percent reductions in land snail shell diameter and weight occurred at 102 mg/kg dw and 135 mg/kg dw HHCB in the soil, respectively (Wang et al., 2015).

Table 3-9. Soil Dwelling Invertebrate Toxicity of HHCB

Test Organism	Hazard Values	Duration	Endpoint	Citation (Study Quality)
Earthworm (*Eisenia fetida*)	489 mg/kg dw	7-day LC50	Mortality	(Chen et al., 2011a) (High)
	392 mg/kg dw	14-day LC50	Mortality	
	50/100 mg/kg dw	28-day NOEC/ LOEC	40% slower growth rate	
	30/50 mg/kg dw	28-day NOEC/ LOEC	30% fewer cocoons per worm produced	
	3/10 mg/kg dw	28-day NOEC/ LOEC	Changes in gene expression of oxidative stress and reproductive biomarkers [b]	
	6.14 µg/cm^2	72-hour LC50 [a]	Adult mortality	(Chen et al., 2011b) (High) (Chen and Zhou, 2012) (High)
	0.3/3.0 µg/cm^2	72-hour NOEC/ LOEC [a]	Changes in gene expression of oxidative stress and reproductive biomarkers [c]	
	105/250 mg/kg dw	8-week NOEC/ LOEC	50% lower food consumption	(Goßmann, 1997) (High)
	45/105 mg/kg dw	8-week NOEC/LOEC	43% lower reproductive rate	
	10/50 mg/kg dw	28-day NOEC/ LOEC	7% increased lipid peroxidase activity	(Liu et al., 2011) (Low)
	188 mg/kg dw	14-day LC50	Mortality	(Wang et al., 2015) (High)
	21 mg/kg dw	14-day EC50	50% fewer cocoons produced	

Test Organism	Hazard Values	Duration	Endpoint	Citation (Study Quality)
	17 mg/kg dw	14-day EC50	50% fewer juveniles produced	
Nematode (*Caenorhabditis elegans*)	1,946 µg/L [d]	24-hour LC50	Mortality	(Mori et al., 2006) (High)
	4,900/9,800 µg/L [d]	24-hour NOEC/ LOEC	Reduced growth	
	9800/19,500 µg/L [d]	24-hour NOEC/ LOEC	Reduced reproduction	
Land snail (*Achatina fulica*)	102 mg/kg dw	14-day EC50	50% smaller shell diameter	(Wang et al., 2015) (High)
	135 mg/kg dw	14-day EC50	50% lower weight	

EC50 = effect concentration at which 50% of test organisms exhibit an effect; NOEC/LOEC = no/lowest-observed-effect-concentration; LC50 = lethal concentration at which 50% of test organisms die
[a] filter paper contact test (OECD No. 207).
[b] increases in gene expression of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and calreticulin (CRT) stress biomarkers and decrease in gene expression of the reproductive gene annetocin (ANN).
[c] increases in gene expression of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) up to 48-hours and down regulation of SOC, CAT, and stress response protein (Hsp70) after 48-hours.
[d] hazard concentration exceeded the HHCB limit of water solubility (1,750 µg/L; (U.S. EPA, 2020)).
Bolded value indicates the hazard effects used for deriving the HHCB hazard threshold for soil dwelling invertebrates (Section 3.3.2.1).

3.3.2.1 Terrestrial Invertebrate Hazard Threshold

EPA reviewed six high quality studies and one low quality study for soil exposure hazard effects to terrestrial invertebrates (Table 3-9). The most sensitive organism for which a clear population-level fitness endpoint could be obtained was the earthworm (Chen et al., 2011a). Chen (2011a) found 30% fewer cocoons were produced with 50 mg/kg dw HHCB in soil compared to earthworms in soil with 30 mg/kg dw HHCB and no HHCB control treatments. The same study found mortality effects from 14-day soil exposures to earthworms at higher HHCB soil concentrations and reported an LC50 of 392 mg/kg dw (Chen et al., 2011a). This study followed standard OECD test guidelines (OECD No. 207 for earthworm acute toxicity and OECD No. 222 for earthworm reproduction tests) for measuring effects of chemical exposure in organic material in soil (OECD, 2016, 1984) with a well-established test species that is widely distributed across the world and is invasive but widespread in the United States (Hendrix and Bohlen, 2002). *Eisenia fetida* earthworms reside above the soil in decaying organic matter (*i.e.,* epigean) and are considered standard representatives of soil fauna (OECD, 2016). EPA used the concentration at the more sensitive endpoint of 30% reduction in reproduction to calculate a COC. EPA calculated the COC as the geometric mean of the LOEC (50 mg/kg dw HHCB) and NOEC (30 mg/kg dw HHCB), equal to 38.7 mg/kg dw, resulting in a hazard threshold to terrestrial invertebrates of 38.7 mg/kg HHCB in soil.

3.3.2.2 Weight of Scientific Evidence for Terrestrial Invertebrate Hazard Threshold

The weight of evidence suggests that HHCB poses hazard effects to soil invertebrates at 38.7 mg/kg HHCB. EPA has robust confidence in the hazard threshold for four reasons. First, the reasonably available database of studies used for this determination includes six high quality studies and one low quality study to determine growth or reproduction effects using standard methods. Second, these studies were conducted on three different species including earthworm (*E. fetida*), nematode (*C. elegans*), and land snail (*A. fulica*) (Table 3-9). Five studies used the standard earthworm *E. fetida*. Third, the studies with *E. fetida* and *A. fulica* found consistent effects within the same order of magnitude of HHCB concentrations. The study that used the nematode *C. elegans* used water HHCB exposures that were

greater than the HHCB limit of water solubility (1,750 µg/L (U.S. EPA, 2020)), making the study less relevant (Mori et al., 2006). Finally, the earthworm studies were conducted with reasonable dose-response designs and results, which enabled precise estimations of effect concentrations. Thus, EPA has robust confidence in the quality, consistency, strength and precision, and relevance of the studies used in determining the hazard threshold for soil invertebrates (38.7 mg/kg HHCB).

3.3.3 Terrestrial Plants

Studies of plant biomass and growth effects of soil amended with HHCB and studies with HHCB added to filter paper using standard methods were conducted on 13 different species including both monocotyledonous and dicotyledonous in seven different families (Table 3-10). Wang (2015) and Spatz (2019) conducted studies with biomass endpoints for HHCB soil addition effects on multiple plant species that ranged from 21-day EC10 values ranging from 3.55 mg/kg dw (IFF, 2019) to 34 mg/kg dw (Wang et al., 2015). The most sensitive plant for which a clear population-level fitness endpoint could be obtained was biomass effects on the rapeseed (*Brassica napus*) with an EC10 value of 3.55 mg/kg dw HHCB (IFF, 2019).

Table 3-10. Terrestrial Plant Toxicity of HHCB

Test Organism	Hazard Values	Endpoint	Duration	Citation (Study Quality)
Corn (*Zea mays*)	93 mg/kg	Root length	21-day EC10	(Wang et al., 2015) (High)
	56 mg/kg	Shoot length		
	59 mg/kg	Wet weight		
Garlic chives (*Allium tuberosum*)	22 mg/kg	Root length		
	15 mg/kg	Shoot length		
	14 mg/kg	Wet weight		
Cucumber (*Cucumis sativus*)	35 mg/kg	Root length		
	18 mg/kg	Shoot length		
	17 mg/kg	Wet weight		
Napa cabbage (*Brassica pekinensis*)	30 mg/kg	Root length		
	23 mg/kg	Shoot length		
	14 mg/kg	Wet weight		
Soybean (*Glycine max*)	41 mg/kg	Root length		
	32 mg/kg	Shoot length		
	31 mg/kg	Wet weight		
Bread wheat (*Triticum aestivum*)	86 mg/kg	Root length		
	39 mg/kg	Shoot length		
	34 mg/kg	Wet weight		
	15,000 µg/L	Root length	21-day EC10	(An et al., 2009) (High)
	30,000 µg/L	Shoot length		
	1,500/3,000 µg/L	Reduced physiological indices [a]	14-day NOEC/LOEC	
	1/10 mg/kg	35% increase in catalase activity	21-day NOEC/LOEC	(Chen and Cai, 2015) (High)

Test Organism	Hazard Values	Endpoint	Duration	Citation (Study Quality)
	1 mg/kg	Reductions in physiological indices [a]	21-day LOEC [b]	
Annual baby's breath (*Gypsophilia elegans*)	>1,750 µg/L	No observed effects on root length up to and including the highest treatment	3-day EC10	(Sinkkonen et al., 2011) (High)
Purslane (*Portoluca oleracea*)	100/1,000 µg/L	7% reduction in root length	4-day NOEC/LOEC	
Rapeseed (*Brassica napus*)	**3.6 mg/kg**	Biomass	21-day EC10	(IFF, 2019) (High)
Soybean (*Glycine max*)	12.3 mg/kg			
Tomato (*Solanum lycopersicum*)	10.3 mg/kg			
Cucumber (*Cucumis sativus*)	5.2 mg/kg			
Common oat (*Avena sativa*)	>1,000 mg/kg			
Onion (*Allium cepa*)	6.78 mg/kg			
Bougainvillea spectabilis	>100 mg/kg	Biomass	40-day	(Zhang et al., 2019) (Low)
Annual baby's breath (*Gypsophilia elegans*)	81.7 µg/L	Shoot length	5-day EC50	(Patama et al., 2019) (Medium)
	55.8 µg/L	Root length		

EC10 = effect concentration at which 10% of test organisms exhibit an effect; NOEC/LOEC = no/lowest observed-effect concentration
[a] Damage to the accumulation of chlorophyll, the synthesis of soluble protein, and the activity of peroxidase, and superoxide dismutase.
[b] Effects were observed at the lowest exposure concentration.
Bolded value indicates the hazard effects used for deriving the HHCB hazard threshold for terrestrial plants (see Section 3.3.3.1).

3.3.3.1 Terrestrial Plant Hazard Threshold

EPA reviewed five high quality studies, one low quality study, and one study not yet rated for soil exposure hazard effects to terrestrial plants (Table 3-10). Wang (2015) and Spatz (2019) conducted studies with biomass endpoints for HHCB soil addition effects on multiple plant species with 21-day EC10 values ranging from 3.6 mg/kg dw (IFF, 2019) to 34 mg/kg dw (Wang et al., 2015). The most sensitive plant for which a clear population-level fitness endpoint could be obtained was biomass effects on the rapeseed with an EC10 value of 3.6 mg/kg dw HHCB (IFF, 2019). EPA calculated the COC as the geometric mean of the EC10 of 3.6 mg/kg dw HHCB, resulting in a Hazard Threshold to terrestrial plants of 3.6 mg/kg HHCB in soil.

3.3.3.2 Weight of Scientific Evidence for Terrestrial Plant Hazard Threshold

The weight of evidence suggests that HHCB poses hazard effects to terrestrial plants at 3.55 mg/kg HHCB. EPA has robust confidence in the hazard threshold for four reasons. First, the reasonably available database of studies used for this determination includes five high quality studies to determine hazard effects on plant biomass using standard methods. Second, these studies were conducted on 13 different species ranging including both monocotyledonous and dicotyledonous in seven different families (Table 3-10). Third, these studies found consistent effects across species within the same order of magnitude of HHCB concentrations. Finally, the plant studies were conducted with reasonable dose-response designs and results, which enabled precise estimations of effect concentrations. Thus, EPA has robust confidence in the quality, consistency, strength and precision, and relevance of the studies used in determining the hazard threshold for terrestrial plants (3.55 mg/kg HHCB).

3.4 Summaries and Conclusions of Environmental Hazard Assessment

3.4.1 Summary of Environmental Hazard Thresholds

After weighing the scientific evidence, EPA selected a toxicity value from the integrated data to use for hazard thresholds to identify potential concerns to aquatic and terrestrial species (Table 3-11).

Table 3-11. Environmental Hazard Thresholds for HHCB

Receptor Group	Exposure Duration	Hazard Threshold (COC or HV)	Assessment Medium	Citation
Aquatic animals	Acute	42.3 µg/L	Water column	From SSD; Section 3.2.1
Aquatic vertebrates	Chronic	9.8 µg/L	Water column	(Croudace et al., 1997)
Sediment-dwelling invertebrates	Chronic	2.4 (mg/kg dw)	Sediment	(IFF, Date Unknown-a)
Terrestrial vertebrates	Chronic	35.0 mg/kg-day	Diet	(IFF, 2021)
Terrestrial invertebrates	Chronic	38.7 mg/kg	Soil	(Chen et al., 2011a)
Terrestrial plants	Chronic	3.6 mg/kg	Soil	(IFF, 2019)
COC = concentration of concern; HV = hazard value				

3.4.2 Summary of Weight of Scientific Evidence for Environmental Hazard Assessment

EPA determined the weight scientific evidence that HHCB poses hazards from acute exposures to aquatic vertebrates and invertebrates (Section 3.2.1.2), and chronic exposures to aquatic vertebrates (Section 3.2.2.3), chronic exposures to sediment-dwelling invertebrates (Section 3.2.4.2), chronic dietary exposure to terrestrial mammals (Section 3.3.1.2), chronic soil exposure to terrestrial invertebrates (Section 3.3.2.2), and chronic soil exposure terrestrial plants (Section 3.3.3.2). Criteria for assessing confidence is described in Appendix E. Overall, EPA has robust confidence in the weight of evidence in these findings.

3.4.3 Environmental Hazard Assessment Conclusions

EPA considered the quality, consistency, strength and precision, biological gradient/dose response, and relevance of the reasonably available data to weigh the scientific evidence in determining the environmental hazards of HHCB. EPA determined that HHCB poses acute exposure hazards to aquatic animals, chronic exposure hazards to aquatic vertebrates, chronic exposure hazards to sediment dwelling invertebrates, chronic dietary exposure hazards to terrestrial mammals, chronic soil exposure hazards to terrestrial invertebrates, and chronic soil hazard effects to terrestrial plants (Table 3-12).

Table 3-12. Environmental Hazard Conclusions for HHCB

Receptor Group	Exposure Duration and Organism Hazard	Hazard Threshold (COC or HV)	Assessment Medium	Citation (Study Quality)
Aquatic animals (Section 3.2.1)	Acute exposure resulting in aquatic vertebrate and invertebrate species mortality	42.3 µg/L	Water column	From SSD
Aquatic vertebrates (Section 3.2.2)	Chronic exposure to aquatic vertebrate species (54% reduction in fish [(*Pimphales promelas*)] growth over 32 days)	9.8 µg/L	Water column	(Croudace et al., 1997) (High)
Sediment-dwelling invertebrates (Section 3.2.4)	Chronic exposure to sediment-dwelling animal species (49% reduction in *Lumbriculus variegatus* reproduction over 28 days)	2.4 mg/kg dw	Sediment	(IFF, Date Unknown-a) (High)
Terrestrial vertebrates (Section 3.3.1)	Chronic dietary exposure to terrestrial mammals (15% lower mammal pup weight over 2 generations)	35.0 mg/kg/day	Diet	(IFF, 2021) (Acceptable/Guideline)
Terrestrial invertebrates (Section 3.3.2)	Chronic exposure to soil invertebrates (30% lower earthworm [*Eisenia fetida*] reproduction over 28 days)	38.7 mg/kg	Soil	(Chen et al., 2011a) (High)
Terrestrial plants (Section 3.3.3)	Chronic exposure in soil to plants (50% lower rapeseed [*Brassica napus*] biomass over 21 days)	3.6 mg/kg	Soil	(IFF, 2019) (High)
COC = concentration of concern; dw = dry weight; HV = hazard value; SSD = species sensitivity distribution				

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APPENDICES

Appendix A CALCULATING DAILY ORAL HUMAN EQUIVALENT DOSES AND HUMAN EQUIVALENT CONCENTRATIONS

For HHCB, all data considered for PODs are obtained from oral animal toxicity studies in rats. Because toxicity values for HHCB are from oral animal studies, EPA must use an extrapolation method to estimate HEDs. The preferred method would be to use chemical-specific information for such an extrapolation. However, no PBPK models or chemical-specific information was identified for HHCB to support a quantitative extrapolation. In the absence of such data, the Agency relied on the guidance from U.S. EPA (2011c), which recommends scaling allometrically across species using the three-quarter power of body weight ($BW^{3/4}$) for oral data. Allometric scaling accounts for differences in physiological and biochemical processes, mostly related to kinetics.

For application of allometric scaling in risk evaluations, EPA uses dosimetric adjustment factors (DAFs), which can be calculated using Equation_Apx A-1.

Equation_Apx A-1. Dosimetric Adjustment Factor

$$DAF = \left(\frac{BW_A}{BW_H}\right)^{1/4}$$

Where:

DAF = Dosimetric adjustment factor (unitless)
BW_A = Body weight of species used in toxicity study (kg)
BW_H = Body weight of adult human (kg)

(U.S. EPA, 2011c) presents DAFs for extrapolation to humans from several species. However, because those DAFs used a human body weight of 70 kg, the Agency has updated the DAFs using a human body weight of 80 kg for the HHCB risk evaluation (U.S. EPA, 2011a). EPA used a body weight of 0.25 kg for rats, as presented in (U.S. EPA, 2011c). The resulting DAF for rats is 0.236.

Use of allometric scaling for oral animal toxicity data to account for differences among species allows EPA to decrease the default intraspecies UF (UF_A) used to set the benchmark MOE; the default value of 10 can be decreased to 3, which accounts for any toxicodynamic differences that are not covered by use of $BW^{3/4}$. Using the appropriate DAF from Equation_Apx A-1, EPA adjusts the POD to obtain the HED using Equation_Apx A-2.

Equation_Apx A-2. Daily Oral Human Equivalent Dose

$$HED_{Daily} = POD_{Daily} \times DAF$$

Where:

HED_{Daily} = Human equivalent dose assuming daily doses (mg/kg-day)
POD_{Daily} = Oral POD assuming daily doses (mg/kg-day)
DAF = Dosimetric adjustment factor (unitless)

EPA assumes similar absorption for the oral and inhalation routes, and no adjustment was made when extrapolating to the inhalation route. For the inhalation route, the Agency extrapolated the daily oral HEDs to inhalation HECs using a human body weight and breathing rate relevant to a continuous exposure of an individual at rest, as follows:

Equation_Apx A-3. Extrapolating from Oral HED to Inhalation HEC

$$HEC_{Daily,continuous} = HED_{Daily} \times \left(\frac{BW_H}{IR_R * ED_C}\right)$$

Where:

$HEC_{Daily,continuous}$	=	Inhalation HEC based on continuous daily exposure (mg/m^3)
HED_{Daily}	=	Oral HED based on daily exposure (mg/kg-day)
BW_H	=	Body weight of adult humans (kg) = 80
IR_R	=	Inhalation rate for an individual at rest (m^3/h) = 0.6125
ED_C	=	Exposure duration for a continuous exposure (h/day) = 24

Based on information from (U.S. EPA, 2011a), EPA assumes an at rest breathing rate of 0.6125 m^3/h. Adjustments for different breathing rates required for individual exposure scenarios were made in the exposure calculations, as needed.

It is often necessary to convert between ppm and mg/m^3 due to variation in concentration reporting in studies and the default units for different OPPT models. Therefore, EPA presents all PODs in equivalents of both units to avoid confusion and errors. Equation_Apx A-4 presents the conversion of the HEC from mg/m^3 to ppm.

Equation_Apx A-4. Converting Units for HECs (mg/m^3 to ppm)

$$X\ ppm = Y\ \frac{mg}{m^3} \times \frac{24.45}{MW}$$

Where:

24.45	=	Molar volume of a gas at standard temperature and pressure (L/mol), default
MW	=	Molecular weight of the chemical (MW of HHCB = 258.41 g/mol)

Appendix B HHCB NON-CANCER HED AND HEC CALCULATIONS FOR INTERMEDIATE AND CHRONIC DURATION EXPOSURES

The intermediate and chronic duration non-cancer POD is based on a $BMDL_5$ of 30 mg/kg-day, and the critical effect is decreased body weight in F1 and F2 offspring. This non-cancer POD is considered protective of effects observed following intermediate and chronic duration exposures to HHCB. EPA used Equation_Apx A-1 to determine a DAF specific to rats (0.236), which was in turn used in the following calculation of the daily HED using Equation_Apx A-2:

$$7.09\ \frac{mg}{kg-day} = 30\frac{mg}{kg-day} \times 0.236$$

EPA then calculated the continuous HEC for an individual at rest using Equation_Apx A-3:

$$38.6\ \frac{mg}{m^3} = 7.09\ \frac{mg}{kg-day} \times \left(\frac{80\ kg}{0.6125\frac{m^3}{hr} * 24\ hr}\right)$$

Equation_Apx A-4 was used to convert the HEC from mg/m^3 to ppm:

$$3.65\ ppm = 38.6\ \frac{mg}{m^3} \times \frac{24.45}{258.41}$$

Appendix C OTHER HAZARD OUTCOMES

This appendix discusses organ systems and outcomes that were not considered for dose-response. These effects have weaker evidence integration conclusions due to inconsistent findings across studies and/or limited dose ranges in the studies that did find an effect.

C.1 Thyroid Effects

C.1.1 Human Evidence

EPA did not identify any epidemiologic studies on thyroid effects for HHCB.

C.1.2 Laboratory Animal Evidence

Studies in Rodents

Effects of HHCB on the thyroid were measured in one EOGRT study and in the associated range-finding study in rats (IFF, 2021, 2020a). These studies are discussed below.

In a simplified reproductive/developmental toxicity screening test (OECD 421) that was used as a range-finding study for the EOGRT, male and female Wistar rats (n = 10 per sex per dose) were exposed to HHCB via the diet at achieved concentrations of 34/38 and 121/134 mg/kg-day HHCB in males/females (IFF, 2020a). Males were dosed for 29 days (*i.e.,* 2 weeks prior to mating, during mating, and up to the day of necropsy). Females were dosed 2 weeks prior to mating, during mating, gestation, and lactation, and up to the day of necropsy (*i.e.,* on LD 21–23 for females that delivered). Notably, this study was a simplified form of OECD 421 and did not include a concurrent control group; instead, the results were compared to historical control data without statistical analysis. Relative thyroid weights were measured in the F0 generation and increased dose-dependently at the low (15%) and high (64%) dose in F0 males relative to historical controls. In F0 females, relative thyroid weights increased at the high dose only (26%) relative to historical controls. Organ weights (including the thyroid) were not measured in F1 offspring. Additionally, other parameters recommended by OECD 421 that are related to thyroid homeostasis (serum levels of T4 and TSH; macroscopic and microscopic observations in the thyroid) were not measured in adults or offspring, and no rationale for omission was provided.

The design and results of the following study are described in more detail in the *Draft Data Evaluation Records for Human Health Hazard for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (U.S. EPA, 2026b). Briefly, in an OECD 443 EOGRT study (IFF, 2021), male and female Wistar rats (n = 25 per sex per dose) were exposed to HHCB at nominal concentrations of 0, 470, 825, or 1650 ppm HHCB in the diet from 10 weeks prior to mating in F0 animals continuously throughout gestation and lactation. In F0 males, this corresponded to average intake of 25.8/45.9/94.1 mg/kg-day at the low/medium/high doses. In F0 females, average intake ranged from 26.8 to 34.4/45.6 to 57.5/91.7 to 116.3 mg/kg-day at the low/medium/high doses depending on whether the timepoint of interest was during pre-mating, gestation, or lactation. At weaning, the F1 generation pups were divided into Cohorts 1A, 1B, and 1C (containing 20 pups/sex/treatment level/cohort) and fed the same dietary concentrations as their parents. In F1 males, this corresponded to an average intake of 33.9/59.6/123.3 mg/kg-day at the low/medium/high doses. In F1 females, this corresponded to an average intake of 27.4 to 35/47.6 to 60/95.2 to 122.1 mg/kg-day at the low/medium/high doses depending on whether the timepoint of interest was during pre-mating, gestation, or lactation.

Thyroid data from the EOGRT study are summarized in Table_Apx C-1 below. F0 adults, a surplus cohort of F1 PND 21 pups, and F1 adults (cohort 1A) were assessed for thyroid weight, histopathology,

serum T4, and serum TSH. Selected culled F1 PND 4 pups also underwent T4 assessment, although raw data were not provided. F1 adults in cohort 1B were also assessed for thyroid weight after mating to produce F2 litters. F2 pups (PND 21) were also assessed for thyroid weight.

In F0 males and females, relative thyroid weight increased concurrently with increases in follicular cell hypertrophy. Specifically, in F0 females, relative thyroid weight increased across all treatment groups (22, 16, and 28% in the low, medium, and high dose groups, respectively) relative to control animals. In F0 males, relative thyroid weight increased dose-dependently and reached statistical significance at the high dose group (18% increase). Incidence of follicular cell hypertrophy increased dose-dependently in both sexes, although the results were not statistically analyzed. Specifically, incidence of follicular hypertrophy increased in the low (1/24), medium (5/24), and high (5/24) doses in males, and in the low (1/24), medium (2/24), and high (5/25) doses in females. Serum TSH did not change significantly or dose-dependently in F0 animals. T4 levels decreased significantly in F0 males-only and reached significance at the middle (25%) and high (26%) dose groups.

In F1 male and female pups in the surplus cohort (PND 21-23), relative thyroid weight increased relative to control animals. Specifically, in F1 males, relative thyroid weight increased significantly at the high dose (7%). In F1 females, relative thyroid weight increased dose-dependently and reached significance at the middle (8%) and high (9%) dose groups. Incidence of thyroid follicular cell hypertrophy did not change relative to controls in either sex. Although serum T4 did not change significantly or dose-dependently in surplus cohort animals, serum TSH increased dose-dependently and reached significance at the high dose group (65%) in males, and the middle (68%) and high (87%) dose groups in females.

In F1 cohort 1A adult males and females (PND 85-93), relative thyroid weight increased relative to control animals. Specifically, in cohort 1A males, relative thyroid weight increased dose dependently and reached significance at the middle (30%) and high (44%) dose groups, respectively. In cohort 1A females, relative thyroid weight increased significantly at the high dose (15%). Incidence of follicular cell hypertrophy increased at the high dose group (3/20) in males, and at the high dose group (2/20) in females. The dose-response relationship could not be determined in females because this outcome was only measured at the high dose. In cohort 1A males, serum T4 decreased dose-dependently and reached significance at the highest dose (34%), and serum TSH increased, reaching significance at the highest dose (82%). In F1 adult females, serum T4 did not change significantly; however, TSH increased dose-dependently, reaching significance at the middle (78%) and high (278%) doses.

In cohort 1B, which consisted of F1 adult males post-mating and dams on LD 21 to 23, relative thyroid weights in males and females increased relative to control animals. Specifically, in cohort 1B males, relative thyroid weight increased dose-dependently and reached significance at the middle (13%) and high (25%) dose groups. In cohort 1B females, relative thyroid weight increased dose-dependently and reached significance at the high (24%) dose group. Incidence of thyroid follicular cell hypertrophy did not change relative to controls in either sex. Thyroid hormones were not measured.

In F2 male and female pups (PND 21–23), thyroid weights and incidences of thyroid follicular cell hypertrophy did not change relative to controls in either sex. T4 and TSH were not measured.

3368 **Table_Apx C-1. Summary of HHCB Studies Evaluating Thyroid Effects in Animals[a]**

Study (Quality Rating)[b]	Generation	Age (Cohort if F1)	Relative liver weight (% of Control)			Relative thyroid weight (% of Control)			T4 (% of Control)			TSH (% of Control)			Absolute Thyroid Weight (% of Control)			Thyroid Follicular Cell Hypertrophy Incidence[c]			
			Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High	Ctrl	Low	Mid	High
Extended one-generation study in rats (IFF, 2021) (Acceptable/Guideline)	F0 Males	After successful mating	6	***8***	***16***	1	6	***18***	−11	***−25***	***−26***	15	−15	16	1	4	***14***	0/25	1/25	5/25	5/25
	F0 Females	LD 22-24	***7***	***3***	***8***	***21***	***16***	***28***	−13	−13	−11	75	33	110	15	16	***24***	0/25	1/24	2/24	5/25
	F1 Males	PND 4 (Culled)	–	–	–	–	–	–	No data provided; reported as similar to control			–	–	–	–	–	–	–	–	–	–
		PND 21–23 (Surplus)	2	1	***7***	2	1	***7***	18	9	22	3	18	***65***	−3	−4	−4	0/10	0/10	0/10	0/10
		PND 85–93 (1A)	5	5	***20***	***25***	***30***	***44***	−7	−16	***−35***	13	4	82	***15***	***16***	***21***	0/20	0/20	0/20	3/20
		After successful mating (1B)	6	7	***11***	2	***13***	***25***	–	–	–	–	–	–	−5	1	4	1/20	1/20	0/20	0/20
	F1 Females	PND 4 (Culled)	–	–	–	–	–	–	No data provided; reported as similar to control			–	–	–	–	–	–	–	–	–	–
		PND 21–23 (Surplus)	3	***8***	***9***	3	***8***	***9***	-7	7	14	28	***68***	***87***	6	5	5	0/10	0/10	0/10	0/10
		PND 85–93 (1A)	0.5	0.2	0.2	5	−2	***15***	−1	−4	−5	−3	***78***	***1,269***	0	−4	8	1/20	–	–	2/20
		LD 21–23 (1B)	5	4	***9***	4	14	***24***	–	–	–	–	–	–	1	11	13	0/19	0/20	0/19	0/19
	F2 Males	PND 21–23				21	1	2	–	–	–	–	–	–	20	−4	−7	0/10	0/10	0/10	0/10
	F2 Females	PND 21–23				26	0.6	−12	–	–	–	–	–	–	26	−5	−20	0/10	0/10	0/10	0/10
Range-finding study in rats (IFF, 2020a) (High)[d]			**Low**		**High**	**Low**		**High**													
	F0 Males		33		48	15		66													
	F0 Females		25		51	−4		26													

LD = lactation day; PND = postnatal day; T4 = thyroxine; TSH = thyroid stimulating hormone
[a] Bolded, italicized numbers denote statistically significant changes. Numbers were rounded to improve readability. Negative numbers reflect decreases relative to control.

Study (Quality Rating)[b]	Generation	Age (Cohort if F1)	Relative liver weight (% of Control)			Relative thyroid weight (% of Control)			T4 (% of Control)			TSH (% of Control)			Absolute Thyroid Weight (% of Control)			Thyroid Follicular Cell Hypertrophy Incidence[c]			
			Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High	Ctrl	Low	Mid	High
[b] Reference was evaluated using the OPP DER format. [c] Total incidence, includes all severities; data were not statistically analyzed. [d] Data from (IFF, 2020a) were not statistically analyzed.																					

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Studies in Aquatic Organisms

In addition to the rodent studies described above, two studies measured effects of HHCB on thyroid-related endpoints in zebrafish larvae (Chae et al., 2023) and in an amphibian metamorphosis assay (Pablos et al., 2016). These are discussed below.

Chae et al. (2023) exposed fertilized zebrafish embryos obtained from a commercial hatchery (250 fertilized embryos per replicate; 3 replicates per treatment group) to nominal concentrations of 0, 0.03, 0.1, 0.3, or 1 mg/L HHCB for 5 days. Average measured concentrations were determined to be much lower than nominal (0.13, 0.34, 0.96, and 3.10 µg/L). Notably, HHCB was dissolved in DMSO, and it is unclear whether control animals received DMSO, given that the other chemical tested in the study was not dissolved in a solvent. The concentration of DMSO was 0.01 percent v/v, and therefore, would not be anticipated to effect T4 levels. Whole-body T4 concentrations in zebrafish homogenates decreased significantly by over 50% (results were reported graphically) across all tested doses relative to control.

In the same study, a separate group of fertilized eggs harvested in-house from sexually mature zebrafish pairs was exposed as described above. Following the 5-day exposure, 4 larvae from each of 3 replicates per dose group were randomly selected for observation of behavioral characteristics (*i.e.,* total distance moved (mm), mean velocity (mm/s), and thigmotaxis) for 60 minutes under different light conditions. Although the total distance moved decreased significantly in the 0.96 µg/L dose group, this outcome lacked dose-response and was not significantly decreased in the highest dose group. No significant, treatment-related changes in thigmotaxis were observed, and data on velocity were not provided. It is unclear why zebrafish from different sources were used for thyroid hormone vs. behavioral measurements in this study.

In an amphibian metamorphosis assay test (OECD 231), *Xenopus laevis* larvae (n = 25 animals per aquaria; 3 aquaria per treatment group) were exposed to 0, 0.05, 0.5, 5, and 50 mg HHCB/kg fish powder dry spiked food daily for 23 days (Pablos et al., 2016). Assay day 23 was selected because this time point would correspond to when a functional thyroid gland can be seen, and endogenous thyroid hormones are detectable.

In tadpoles, developmental acceleration occurred on day 14 in the 50 mg/kg-day dose group, although this effect did not persist at day 23. Mortality and malformations were assessed daily and were unaffected. No significant effects on whole body length (measured on day 14), snout to vent length (measured on day 23), hind limb length (measured on days 14 and 23), or body weight (measured on day 23 and in metamorphosized frogs) were reported. In metamorphosized frogs, the snout-to-vent-length increased in all treatment groups relative to control when results were pooled across aquaria, reaching statistical significance starting at the 0.5 mg/kg food dose group; however, there were no statistically significant differences between control animals and each dose group in individual aquaria.

Dose-dependent increases in the incidence and grade of thyroid histopathology (hypertrophy, hyperplasia, and/or colloid) occurred in the two highest dose groups for both tadpoles and metamorphosized frogs, although these were not statistically analyzed and were described qualitatively as percentages out of 6 randomly selected specimens in each dose group. On day 23 in tadpoles, the percentage of tadpoles with hypertrophy, hyperplasia, and/or increased colloid increased dose-dependently. Specifically, in the tadpoles dosed with the 5 mg/kg food, 33.4% had grade 1 (mild) hypertrophy, hyperplasia, and vacuolated colloid. In tadpoles dosed with 50 mg/kg food, 66.6% had grade 1 hypertrophy and hyperplasia and 33.4 had grade 2 (moderate) hypertrophy. In metamorphosized frogs, the percentage of animals with hypertrophy, hyperplasia, and/or increased colloid also increased dose dependently. Specifically, in frogs from the 5 mg/kg dose group, 50% had grade 1 colloid; In frogs

from the 50 mg/kg dose group, 83.3% had grade 3 colloid and 16.7% had grade 2 hypertrophy. No remarkable changes were noted in thyroids of control tadpoles and metamorphosized frogs.

C.1.3 Mechanistic and Supporting Evidence

Three *in vitro* studies (Cavanagh et al., 2018; Schnell et al., 2009; Mori et al., 2007) provide insight into the mechanisms that potentially influence effects on the thyroid noted in laboratory animal studies. These studies are discussed below.

HHCB was not an agonist or antagonist for the human thyroid hormone receptor in a Chinese hamster cell reporter gene assay (Mori et al., 2007). Furthermore, according to the T4-transthyretin (TTR) competitive fluorescence displacement method, HHCB does not competitively bind TTR, which is a major binding protein for T4 in the blood (Cavanagh et al., 2018).

Alternatively, decreased T4 and related thyroid effects (increased TSH, increased thyroid weight, and histopathology) noted in laboratory animals could be driven by increased clearance of T4 due to induction of liver enzymes (Capen, 1992). To date, one study explored the effect of HHCB exposure on phase II enzyme activities and found that 1mM HHCB significantly increased carp liver microsomal activity of UDP-glucuronosyltransferase (the main enzyme involved in T4 clearance) toward α-naphthol (which is a phenolic compound like T4) to 200% of control (Schnell et al., 2009). Furthermore, given that increased liver weights can be an adaptive response to the induction of enzymes in the liver, increases in liver weights noted in the EOGRT study in rats (IFF, 2021) additionally support a link between decreased T4 and enzyme induction in the liver.

C.1.4 Evidence Integration Summary

Two studies in rats (IFF, 2021, 2020a) and two studies in aquatic organisms (Chae et al., 2023; Pablos et al., 2016) provide evidence that HHCB produces effects that are associated with hypothyroidism, including direct effects (decreased T4) and indirect effects associated with an adaptive response to decreased thyroid hormones (increased TSH, increased thyroid weight, and thyroid follicular cell hypertrophy). Although these findings suggest a potential effect on thyroid homeostasis, the adversity of these endpoints is uncertain given the absence of effects on thyroid hormones (T4) in dams or pups in multi-generational rodent toxicity studies, and on thyroid-mediated apical endpoints, such as neurodevelopmental outcomes across all four studies. Furthermore, the human-relevance of this outcome is questionable given concurrent increases in liver weight that are suggestive of a rodent-dependent mechanism by which liver enzyme induction increases thyroid hormone clearance. This is discussed in more detail below.

The two studies that measured thyroid outcomes in developmentally exposed zebrafish and frogs report decreased T4 and histopathological findings in the thyroid, respectively, that were considered not adverse in isolation given that apical, thyroid-mediated neurobehavioral and developmental endpoints remained unaltered. Specifically, although total body T4 decreased by over 50% (from almost 300 ng/g tissue in controls to under 100 ng/g tissue across all HHCB treatment groups) in zebrafish larvae exposed for 5 days via ambient water, neurobehavioral outcomes that are known to be regulated by thyroid hormones remained unchanged, including locomotive fitness (measured by total distance moved under different lighting conditions) and anxiety (measured by thigmotaxis) (Chae et al., 2023). Additionally, although the percentage of animals with mild or moderate thyroid histopathology (hypertrophy, hyperplasia, and/or increased colloid) increased in tadpoles and metamorphosized frogs exposed to HHCB via the diet for 23 days, thyroid hormones were not measured in this study and all thyroid hormone-mediated developmental endpoints measured (malformations, developmental staging, timing to metamorphosis, body weight, and length) remained unchanged (Pablos et al., 2016).

Although T4 also decreased in the rodent EOGRT study, this change only occurred in adult males for both the F0 and F1 generations; T4 levels were not significantly altered in F1 pups of either sex at PNDs 4 or 21, or in adult F0 or F1 females. This suggests that decreased T4 in F1 males may have been caused by direct exposure post-weaning rather than due to developmental exposure. These outcomes in adult F1 male rats were considered non-adverse in isolation; concurrent histopathological findings were graded as "mild" or "moderate" follicular cell hypertrophy, and no additional adverse outcomes were noted in these animals that could be related to decreased T4 during adulthood (*e.g.,* effects on male reproductive parameters).

Although T4 did not significantly decrease in other populations in the EOGRT, additional outcomes that are associated with an adaptive response to decreased thyroid hormones (increased TSH, thyroid weight, and/or hepatocellular hypertrophy) significantly changed in F0 dams and in F1 pups. Specifically, in F0 animals, thyroid weights increased consistently in males and females across both the EOGRT and range-finding study; however, this effect is of questionable adversity by itself. Although incidence of thyroid follicular cell hypertrophy also increased in F0 males and females in the EOGRT study (this was not measured in the range-finding study), they were graded as "minimal" or "mild" and were not accompanied by any other histopathological findings in the thyroid. In F1 animals in the EOGRT, relative thyroid weights increased significantly and dose-dependently in male and female PND 21 pups and persisted in both cohorts (1A and 1B) of adult F1 animals. Additionally, TSH increased significantly in F1 male and female PND 21 pups and persisted in adult F1 females. These findings were of questionable adversity because there were no concurrent effects on thyroid histopathology (other than "mild" follicular cell hypertrophy in adult males), or behavior from clinical observations.

Given that maternal and fetal thyroid hormones (T3 and T4) are crucial for proper neurodevelopment (Dierichs et al., 2025), it is important to consider whether the effects described above in F1 pups (increased TSH and thyroid weights at PND 21) are indicative of an adaptive response to insufficient levels of maternal or fetal thyroid hormones, and in turn, of the potential for developmental neurotoxicity. T4 was unaltered in F0 dams, in PND 4 F1 pups, and in PND 21 F1 pups, suggesting that maternal and fetal thyroid hormones were not altered. Furthermore, although a developmental neurotoxicity cohort was not included in the EOGRT, no adverse behavioral effects were noted in clinical observations in F1 pups during lactation or post-weaning, and no macroscopic abnormalities were noted in the brains of F1 pups on PND 21. This is additionally consistent with a study in zebrafish, which found that HHCB exposure did not alter neurodevelopmental endpoints. Therefore, in lieu of information from a developmental neurotoxicity cohort, the available data suggest that increased TSH and thyroid weights in F1 offspring were not adverse in isolation, and that neurodevelopment was not impacted in F1 offspring.

According to Table_Apx C-1, in both the EOGRT study and range-finding study, thyroid effects co-occurred with liver weight increases in both sexes for every generation and age that were evaluated except for F1 adult females in cohort 1A in the EOGRT study. This suggests that thyroid effects noted in directly and developmentally exposed animals could be driven by increased clearance of T4 due to the induction of liver enzymes (Capen, 1992). This potential mechanism is further supported by an *in vitro* study that found that HHCB increased carp liver microsomal activity of UDP-glucuronosyltransferase (the main enzyme involved in T4 clearance) toward α-naphthol (which is a phenolic compound like T4) (Schnell et al., 2009). Importantly, rodents are more sensitive to this mechanism of than humans due to the shorter plasma half-life of T4 in rodents and due to the considerable differences between species in the transport proteins for thyroid hormones (Capen, 1992). Therefore, developmental thyroid effects in the available studies have questionable human relevance.

Given the uncertainties regarding the adversity and human-relevance of the thyroid effects reported in the available studies and given that decreased offspring bodyweights are protective of these effects, EPA is not further considering this endpoint for dose-response analysis or for use in estimating risk to human health.

C.2 Liver Toxicity

Considerations for Interpretation of Hepatic Effects:
Consistent with previous guidances (Hall et al., 2012; U.S. EPA, 2002a), EPA considered hepatocellular hypertrophy and corresponding increases in liver size and weight to be adaptive non-adverse responses, where these observations were not accompanied by treatment-related, biologically significant changes (*i.e.,* 2- to 3-fold) in clinical chemistry markers of liver toxicity; that is, decreased albumin; or increased alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma glutamyltransferase (GGT), bilirubin, cholesterol) and/or histopathology in liver indicative of an adverse response (*e.g.,* hyperplasia, degeneration, necrosis, inflammation).

C.2.1 Human Evidence

EPA did not identify any epidemiologic studies on liver injury for HHCB.

C.2.2 Laboratory Animal Evidence

Liver effects of HHCB have been reported in orally and dermally exposed rats and mice. Available oral studies include one EOGRT study and the associated range-finding study in rats (IFF, 2021, 2020a), one 2-week uterotrophic assay in mice (Seinen et al., 1999), and one 90-day oral repeated-dose toxicity study in rats (Api and Ford, 1999). Available dermal studies include one 26-week subacute dermal toxicity study in rats (IFF, Date Unknown-c). These studies are summarized in Table_Apx C-2 and are discussed below.

The design and results of the following study are described in more detail in *Draft Data Evaluation Records for Human Health Hazard for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB)* (U.S. EPA, 2026b). Briefly, in an OECD 443 EOGRT study (IFF, 2021), male and female Wistar rats (n = 25 per sex per dose) were exposed to HHCB at nominal concentrations of 0, 470, 825, or 1650 ppm HHCB in the diet from 10 weeks prior to mating in F0 animals continuously throughout gestation and lactation. In F0 males, this corresponded to average intake of 25.8/45.9/94/1 mg/kg-day at the low/medium/high doses. In F0 females, average intake ranged from 26.8 to 34.4/45.6 to 57.5/91.7 to 116.3 mg/kg-day at the low/medium/high doses depending on pre-mating vs. gestation, lactation. At weaning, the F1 generation pups were divided into Cohorts 1A, 1B, and 1C (containing 20 pups/sex/treatment level/cohort) and fed the same dietary concentrations as their parents. In F1 males, this corresponded to an average intake of 33.9/59.6/123.3 mg/kg-day at the low/medium/high doses. In F1 females, this corresponded to average intake of 27.4 to 35/47.6 to 60/95.2 to 122.1 mg/kg-day at the low/medium/high doses depending on pre-mating vs. gestation vs. lactation. Cohort 1A animals were assessed for clinical pathology, time between vaginal patency and onset of estrus, estrous cycle data, differential ovarian follicle counts, sperm parameters, and splenic lymphocyte subpopulation analysis, and were terminated on PND 85 to 93.

Cohort 1B offspring were mated to produce F2 litters, assessed for reproductive parameters, and terminated after mating (males) or on LD 21 to 23 (females). F1 offspring from all cohorts were assessed for vaginal opening and preputial separation, and Cohort 1C animals were terminated after positive identification of these landmarks. Some unselected F1 offspring were assigned to a surplus cohort for assessment of thyroid-related hormones and organ weights (on PND 21), with termination on

PND 21 to 23. Selected culled PND 4 pups were also used for hormone assessment. In addition to standard assessment of litter parameters for F1 and F2 offspring, F2 pups were also examined for anogenital distance (AGD) on PND 1 and were terminated on PND 21 to 23.

The following liver effects were noted in the F0 generation: In F0 animals, liver weight increased dose-dependently and reached significance at the middle (7%) and high (16%) doses in males, and was significantly affected at the low (7%), middle (3%), and high (8%) doses in females. No corresponding changes in histopathology or clinical chemistry markers were observed except for increased ALP at the high dose (42%) in males. Given that serum ALP can increase due to factors other than liver injury and isoenzymes from liver, bone, and kidney share a common protein structure, this endpoint in isolation is not necessarily indicative of liver-specific effects (Dufour et al., 2000). Furthermore, increased ALP did not meet the two-fold threshold for adversity. Therefore, these findings were considered non-adverse.

In F1 pups at PND 21, (surplus cohort), relative liver weight increased significantly at the high dose (7%) in males and dose-dependently in the middle (8%) and high (9%) doses in females. Liver histopathology and clinical chemistry were not measured in this cohort, and these effects were considered non-adverse given the low magnitude of change. Post-weaning, liver weights were still increased in F1 animals (cohort 1A and 1B). Specifically, in cohort 1A, liver weight increased significantly at the high dose (20%) in males, but not in females. In cohort 1B, relative liver weight increased significantly at the high dose (11%) in males and at the high dose (9%) in females. Incidence of centrilobular hypertrophy also increased at the high dose (6/20) in males, but not females (0/20). Notably, this was likely not adverse due to no reports of hyperplasia, degeneration, necrosis, or inflammation in liver. Serum ALT increased significantly at the high dose (31%) in F1 females-only, but this did not meet the two-fold threshold for adversity.

In F2 pups on PND 21, liver weights dose-dependently increased and reached significance at the middle (8%) and high dose (14%) in males, and at the middle (9%) and high (18%) dose in females. No histopathological lesions were reported in the livers, and clinical chemistry were not measured in this cohort; therefore, these effects were considered non-adverse given the low magnitude of change.

In a simplified reproductive/developmental toxicity screening test (OECD 421) that was used as a range-finding study for the EOGRT, male and female Wistar rats (n = 10 per sex per dose) were exposed to HHCB via the diet at achieved concentrations of 34/38 and 121/134 mg/kg-day HHCB in males/females (IFF, 2020a). Males were dosed for 29 days (*i.e.,* 2 weeks prior to mating, during mating, and up to the day of necropsy). Females were dosed 2 weeks prior to mating, during mating, gestation, and lactation, and up to the day of necropsy (*i.e.,* on LD 21–23 for females that delivered). Notably, this study did not include a concurrent control group; instead, the results were compared to historical control data. The following results relevant to liver toxicity were noted in F0 animals: Increased relative liver weight in both sexes that was of uncertain biological significance because the authors did not measure histopathology or clinical markers of liver toxicity.

In a mouse uterotrophic assay (Seinen et al., 1999), 21-day old female Balb/c mice (n = 6 per dose) were exposed to 0, 6, or 40 mg/kg-day via the diet for 2 weeks. Relative liver weight increased in females (males were not included in the study); however, this was of uncertain biological significance because the authors did not measure histopathology or clinical markers of liver toxicity.

In a non-guideline subacute dermal toxicity study, Galaxolide 50 diluted in ethanol was applied daily to the shaved backs of SD rats (n = 15 males and 35–38 females per dose) at concentrations of 0, 50, 100, or 200 mg/kg-day of for 26 weeks (IFF, Date Unknown-c). Given that Galaxolide 50 is a mixture of

65% HHCB, the equivalent concentrations of HHCB were 0, 32.5, 65, and 130 mg/kg-day. A vehicle control group received ethanol at a volume equal to the largest volume administered to a test group. At the highest doses (65 and 130 mg/kg-day) some rats displayed scabbed areas and appearance of a white or brown crusty material. No statistically significant effects were observed on body weights, hematology, biochemical parameters or urinalysis. Organs were unaffected except for an increase in relative liver weight in females at week 26 (11 and 23% in the 65 and 130 mg/kg-day dose groups, respectively) and kidney weights (37% in males receiving the highest dose). No histopathological defects were noted in liver or kidney. Notably, this study was not designed to distinguish effects of HHCB from those of DEP and lacked a description of measures taken to prevent ingestion of the test material.

3619 **Table_Apx C-2. Summary of HHCB Studies Evaluating Effects on the Liver in Animals**

Reference	Study Description	NOEL/LOEL (mg/kg-day)[a]	Liver Weight	Histopathology	Clinical Chemistry	Study Quality Rating
(IFF, 2021)	Male and female Wistar rats (n = 25 per sex per dose); 0,470, 825, or 1,650 ppm in the diet; 10 weeks prior to mating in F0 animals through PND 21-23 in F2 pups Achieved doses in mg/kg-day in the low/medium/high dose groups were: In F0 males: 25.8/ 45.9/ 94.1 In F0 females: 26.8–34.4/ 45.6–57.5/ 91.7–116.3 In F1 males: 33.9/ 59.6/ 123.3 In F1 females: 27.4–35/ 47.6–60/ 95.2–122.1 mg/kg-day EOGRT study (OECD 443)	In F0 adults				Acceptable/Guideline[a]
		26.8/ 45.6	↑ Relative liver weight in males (7-16%) and females (3-8%)	No histopathologic findings in males or females	ALP increased at the highest dose in males-only (42%); AST, ALT, bilirubin, and cholesterol did not change in males or females; Albumin did not decrease	
		In F1 pups on PND 21 (surplus cohort)				
		26.8/ 45.6	↑ Relative liver weight in males (7%) and females (8-9%)	No histopathologic findings in males or females	Not measured	
		In F1 adults (cohort 1A)				
		45.6/ 91.7	↑ Relative liver weight (20%) in males-only	↑ Centrilobular hypertrophy (6/20 animals) in males-only	↑ ALT in females -only (31%); AST, ALP, bilirubin, and cholesterol did not increase; Albumin did not decrease	
		In F1 adults (cohort 1B)				
		45.6/ 91.7	↑ Relative liver weight in males (11%) and females (9%)	Not measured	Not measured	
		In F2 pups (PND 21)				
		27.4/ 47.6	↑ relative liver weight in males (8-14%) and females (9-18%)	Not measured	Not measured	

Reference	Study Description	NOEL/LOEL (mg/kg-day)[a]	Liver Weight	Histopathology	Clinical Chemistry	Study Quality Rating
(IFF, 2020a)	Wistar rats (n = 10 per sex per dose) exposed via diet to 34/38 and 121/134 mg/kg-day for males/females. Males were dosed for 29 days (*i.e.,* 2 weeks prior to mating, during mating, and up to the day of necropsy). Females were dosed 2 weeks prior to mating, during mating, gestation, and lactation, and up to the day of necropsy (*i.e.,* on LD 21 to 23 for females that delivered). OECD 421	ND/ 34	↑ Relative liver weight relative to historical controls in F0 males (33–48%) and females (25–51%)	Not measured	Not measured	High
(Seinen et al., 1999)	Uterotrophic assay in 21-day old female Balb/c mice (n = 6 per dose) exposed via diet to 0, 6, or 40 mg/kg-day for 2 weeks.	ND/6	↑ Relative liver weight (8–22%)	Not measured	Not measured	Acceptable/Non-guideline[a]
(Api and Ford, 1999)	SD rats (n = 15 per sex per dose) exposed via diet to 0, 5, 15, or 150 mg/kg-day for 13 weeks. OECD 408	150/ND	No change in relative liver weight in males or females	No abnormalities observed at necropsy or in histopathology	Not measured	Medium
(IFF, Date Unknown-c)	SD rats (n = 15 males and 35–38 females per dose) exposed dermally to 0, 50, 100, or 200 mg/kg-day of Galaxolide 50 (65% HHCB in DEP) diluted in ethanol for 26 weeks. This equates to	32.5/65	Increased relative liver weight was observed in females but not males at the two highest doses (11% and 23%, respectively)	No histopathological findings in males or females	No changes in bilirubin or ALP	High

Reference	Study Description	NOEL/LOEL (mg/kg-day)[a]	Liver Weight	Histopathology	Clinical Chemistry	Study Quality Rating
	32.5, 65, and 130 mg/kg-day HHCB.					

ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; LOEL = lowest-observed-effect level; ND = not determined; NOEL = no-observed-effect level; OECD = Organization for Economic Co-operation and Development; SD = Sprague-Dawley (rats)
[a] Reference was evaluated using the OPP DER format.

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C.2.3 Mechanistic and Supporting Evidence

None of the available studies investigated peroxisome proliferation, oxidative stress, inflammation, apoptosis, or other mechanisms of liver injury due to HHCB exposure.

C.2.4 Evidence Integration Summary

Although no epidemiological studies measure effects of HHCB in the liver, laboratory animal studies provide evidence of dose-dependent increases in liver weight in response to HHCB. However, these effects are inconsistent across studies and are not considered adverse. This is discussed in more detail below.

Across the available studies that investigated liver toxicity endpoints in directly exposed adult animals, increased liver weights differ across sexes, with no clear pattern of effect. Specifically, while a mouse uterotrophic assay involving oral exposure for 2 weeks (Seinen et al., 1999) found dose-dependent increases in liver weights in females starting at the lowest tested dose (6 mg/kg-day), an EOGRT study found effects in F0 males but not females that did not reach significance until 94 mg/kg-day. Furthermore, a 90-day repeated dose oral study in rats (Api and Ford, 1999) did not find any significant changes in liver weights in either sex at doses as high as 150 mg/kg-day, whereas a 26-week dermal study in rats (IFF, Date Unknown-c) found significantly increased liver weights in females-only starting at 65 mg/kg-day.

In addition to these inconsistencies across adult males and females and across studies, none of the available studies in adult animals reported concurrent changes in clinical markers of liver toxicity or adverse histopathological findings alongside liver weight increases. Specifically, 2 of the 4 studies that observed increased liver weights found no concurrent changes in these additional parameters at doses as high as 100 and 91.7 mg/kg-day, respectively (IFF, Date Unknown-b, 2021). The remaining two studies did not measure additional endpoints related to liver toxicity (IFF, 2020a; Seinen et al., 1999). Given the information available, the increased liver weights noted in these studies are not indicative of an adverse response according to guidance from EPA (Hall et al., 2012; U.S. EPA, 2002a).

Only one of the available studies (the EOGRT study) measured liver endpoints in F1 and F2 offspring after developmental exposure; therefore, developmental effects on the liver cannot be analyzed for consistency across studies. This study reported dose-dependent increases in liver weights for both F1 and F2 offspring; however, the adversity of this outcome is unclear given that liver histopathology and clinical chemistry markers either changed in one sex or were not measured. Specifically, in the F1 generation, liver weights increased dose-dependently (ranging from 7–9%) in F1 pups at PND 21, and these increases persisted into adulthood for both sexes (ranging from 9–20%). Although mild centrilobular hypertrophy occurred at the highest tested dose in 6 out of 20 males, no histopathological findings were reported in females. Furthermore, concurrent changes in clinical chemistry markers (ALT, but not AST or ALP) occurred in females-only. In F2 pups, although liver weights decreased dose-dependently in both sexes (ranging from 8–18%), no histopathological lesions were reported, and clinical chemistry parameters were not measured. Furthermore, because these effects occurred at the high dose in F1 animals (F0 maternal intake of 91.7 mg/kg-day) and at the middle dose in F2 animals (F0 maternal intake of 45.6 mg/kg-day), they are not more sensitive than effects on body weight discussed in Section 2.4.3.

Due to these inconsistencies and the lack of adversity in the available laboratory animal studies, the Agency is not further considering liver effects for dose-response assessment.

C.3 Eye Irritation

C.3.1 Human Evidence

EPA did not identify any studies evaluating the effects of HHCB on eye irritation in humans.

C.3.2 Laboratory Animal Evidence

Available studies evaluating the potential of HHCB to irritate the eyes in animals include three acute dermal irritation/corrosion tests in rabbits (IFF, 1975b, 1973a, 1963). These studies are summarized in Table_Apx C-3 and are discussed below.

In the first acute eye irritation/corrosion test in rabbits by IFF (1973a), albino rabbits (sex and strain not specified; n = 3 per treatment group) were exposed to 50% Galaxolide-50 (final concentration of 35% HHCB in DEP and alcohol SDA 39C) in the right eye. Vehicle control animals were treated with 100% alcohol SDA 39C in the right eye. The untreated left eye of each animal served as its own control. Eyes were evaluated according to the Draize method every 24 hours for 4 days and then again on the 7th day. The study authors reported mild, reversible conjunctival irritation in all three animals and corneal involvement in one animal that cleared by day 7. Mild, reversible conjunctival irritation was also noted in the vehicle control animals and cleared on day 4. The authors stated that this study was conducted in general compliance with OECD 405. Results in the left (negative control) eye were not provided for this study.

In a second acute eye irritation/corrosion test in rabbits by IFF (1975b), albino rabbits (sex and strain not specified; n = 3 per treatment group) were exposed to 100% Galaxolide-50 (final concentration of 70% HHCB in DEP), 50% alcohol SDA 39C in water, or 100% alcohol SDA 39C in the right eye. The untreated left eye of each animal served as its own control. Eyes were evaluated according to the Draize method every 24 hours for 4 days and then again on the 7th day. The study authors reported no irritation in Galaxolide 50-treated animals. Mild, reversible conjunctival irritation was noted in both alcohol SDA 39C-treated groups. The authors stated that this study was conducted in general compliance with OECD 405. Results in the left (negative control) eye were not provided for this study.

In a third acute eye irritation/corrosion test in rabbits by IFF (1963), albino rabbits (sex and strain not specified; n = 3 per treatment group) were exposed to 3.75% HHCB diluted in alcohol SDA 39C in the right eye. The untreated left eye of each animal served as its own control. Eyes were evaluated according to the Draize method every 24 hours for 4 days and then again on the 7th day. Mild, reversible conjunctival irritation was noted that cleared by the 7th day of observation. Although the authors stated that this study was conducted in general compliance with OECD 405, there were limitations including a lack of reporting for negative control (left eye) and vehicle control animals. A summary table of eye irritation studies by IFF at the end of the report mentioned that mild conjunctival irritation and corneal involvement that lasted to day 7 occurred in one rabbit treated with 97.5% alcohol SDA 39C, which is an appropriate vehicle control for the present study; however, the raw data for these vehicle-treated animals were not provided in the report.

Table_Apx C-3. Summary of HHCB Studies Evaluating Eye Irritation in Animals

Reference	Study Description	Effects	Study Quality Rating
(IFF, 1973a)	Albino rabbits (sex and strain not specified; n = 3 per treatment group) exposed to 50% Galaxolide-50 (final concentration of 35% HHCB in DEP and alcohol SDA 39C) or 100% alcohol SDA 39C in the right eye. The untreated left eye of each animal served as its own control. Eyes evaluated according to the Draize method every 24 hours for 4 days and then again on the 7th day.	Mild, reversible conjunctival irritation in 1 animal that cleared by day 7 and also appeared in vehicle controls animals; mild corneal involvement in 1 animal	Medium
(IFF, 1975b)	Albino rabbits (sex and strain not specified; n = 3 per treatment group) exposed to 100% Galaxolide-50 (final concentration of 70% HHCB in DEP), 50% alcohol SDA 39Cin water, or 100% alcohol SDA 39C in the right eye. The untreated left eye of each animal served as its own control. Eyes evaluated according to the Draize method every 24 hours for 4 days and then again on the 7th day.	No irritation in Galaxolide 50-treated animals	Medium
(IFF, 1963)	Albino rabbits (sex and strain not specified; n = 3 per treatment group) exposed to 3.75% HHCB in alcohol SDA 39C in the right eye. The untreated left eye of each animal served as its own control. Eyes evaluated according to the Draize method every 24 hours for 4 days and then again on the 7th day.	Mild, reversible conjunctival irritation that cleared by day 7, and which was reported to also appear in vehicle control animals	Medium

C.3.3 Mechanistic and Supporting Evidence

EPA did not identify any reasonably available information that provides mechanistic support for effects of HHCB on eye irritation.

C.3.4 Evidence Integration Summary

Acute eye irritation/corrosion studies in rabbits found mild, reversible conjunctival irritation only in cases where HHCB was diluted in alcohol SDA 39C; No effects were seen in an identical test using a higher concentration of HHCB (70% HHCB diluted in DEP) that was not further diluted in alcohol SDA 39C. Given that alcohol SDA 39C is a GHS category 2A eye irritant ("causes serious eye irritation"), and that similar findings of mild, reversible conjunctival irritation were reported in animals treated with alcohol SDA 39C alone, the effects seen at lower doses were potentially due to the diluent rather than to HHCB. Consistent with other assessments (NICNAS, 2019; OCSPP, 2014; ECB, 2008a, b), EPA concluded that HHCB is not an eye irritant and did not carry this endpoint forward for dose-response or human health risk characterization.

Appendix D SPECIES SENSITIVITY DISTRIBUTION

An SSD was derived using only acute duration exposure studies that calculated LC50s. The SSD Toolbox is a resource that can fit SSDs to environmental hazard data (Etterson, 2020a). It runs on Matlab 2018b (9.5) for Windows 64 bit. For this draft HHCB risk evaluation, EPA created one SSD with the SSD Toolbox Version 1.1 to evaluate acute aquatic vertebrate and invertebrate toxicity. The use of this probabilistic approach increases confidence in the hazard threshold identification as it is a more data-driven way of accounting for uncertainty. For the acute SSD, acute exposure hazard data for aquatic vertebrates and invertebrates were curated to prioritize study quality and to assure comparability between toxicity values. For example, the empirical dataset included only LC50s for high and medium quality acute duration assays that measured mortality for aquatic vertebrates and invertebrates (Table_Apx D-1).

With this dataset, the SSD Toolbox was used to apply a variety of algorithms to fit and visualize SSDs with different distributions. An HC05 was calculated for each. The SSD Toolbox output contained several methods for choosing an appropriate distribution and fitting method, including goodness-of-fit, standard error, and sample-size corrected Akaike Information Criterion (AICc, (Burnham and Anderson, 2002)). Using the maximum likelihood fitting method, most p-values for goodness-of-fit were less than 0.05, showing no evidence of lack of fit. The distribution and model with the lowest AICc value, and therefore the best fit for the data was the Weibull Distribution (Table_Apx D-2). Because numerical methods may lack statistical power for small sample sizes, a visual inspection of the data was also used to assess goodness-of-fit using Q-Q plots (Figure_Apx D-1). For Q-Q plots, the horizontal axis gives the empirical quantiles while the vertical axis gives the predicted quantiles (from the fitted distribution). The Q-Q plot demonstrates a good model fit with the data points near the line across the data distribution. Q-Q plots were visually used to assess the goodness-of-fit for the distributions with the Weibull distribution demonstrating the best fit near the low end of the distribution, which is the region from which the HC05 is derived. The results for this model (Figure 3-1) predicted 5% of the species (HC05) to have their LC50s exceeded at 111 µg/L HHCB (42 to 219 µg/L 95% CI).

Table_Apx D-1. SSD Model Input for HHCB Acute Exposure Toxicity in Aquatic Vertebrates and Invertebrates – Empirical Data

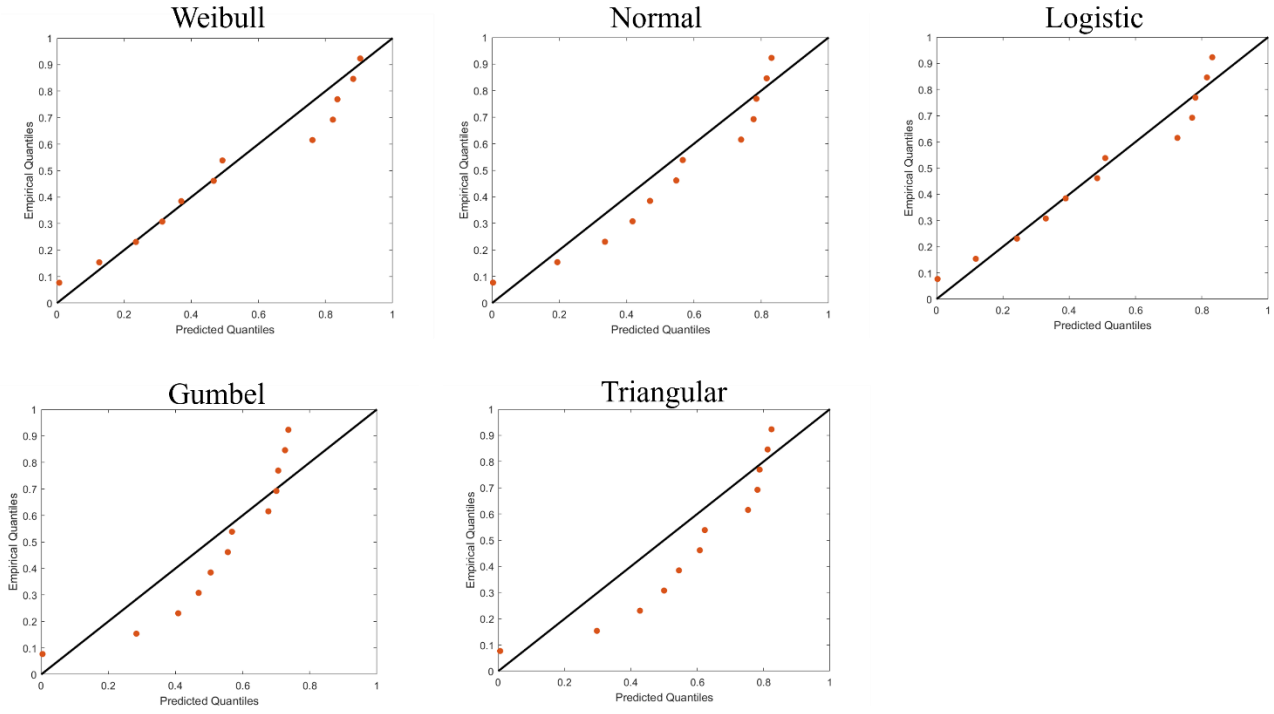
Species	Description	Acute Toxicity Value LC50 (µg/L)	Study
Rana nigromaculata	Vertebrate	35	(Fan et al., 2019)
Daphnia magna	Invertebrate	194	(Chen et al., 2015)
Oryzias latipes	Vertebrate	950	(Yamauchi et al., 2008)
Misgurnus anguillicaudatus	Vertebrate	491	(Fan et al., 2019)
Gobiocypris rarus	Vertebrate	754	(Fan et al., 2019)
Oryzia latipes sinensis	Vertebrate	839	(Fan et al., 2019)
Macrobrachium nipponense	Invertebrate	350	(Fan et al., 2019)
Chironomus plumosus	Invertebrate	861	(Fan et al., 2019)
Chironomus riparius	Invertebrate	288	(Artola-Garicano et al., 2003)
Lampsilis cardium	Invertebrate	999	(Gooding et al., 2006)

Species	Description	Acute Toxicity Value LC50 (µg/L)	Study
Lumbriculus variegatus	Invertebrate	394	(Artola-Garicano et al., 2003)
Acartia tonsa	Invertebrate	470	(Wollenberger et al., 2003)

Table_Apx D-2. SSD Model a Predictions for Acute HHCB Exposure Toxicity to Aquatic Vertebrates

Distribution[b]	HC05 (µg/L)	Akaike Information Criterion	P-Value
Weibull	**111**	**177**	**0.45**
Logistic	126	180	0.61
Normal	95	182	0.22
Triangular	59	183	0.02
Gumbel	71	189	0.01

[a] The SSD was generated using SSD Toolbox v1.1.
[b] The model with the lowest AICc value, and therefore the best model fit, is **bolded** in this table.



Figure_Apx D-1. Q-Q Plots of Species Sensitivity Distribution Model Fits

Appendix E RUBRIC FOR WEIGHT OF SCIENTIFIC EVIDENCE

The weight of scientific evidence fundamentally means that the evidence is weighed (*i.e.,* ranked) and weighted (*i.e.,* a piece or set of evidence or uncertainty may have more importance or influence in the result than another). Based on the weight of scientific evidence and uncertainties, a confidence statement was developed that qualitatively ranks (*i.e.,* robust, moderate, slight, or indeterminate) the confidence in the hazard threshold. The qualitative confidence levels are described below.

The evidence considerations and criteria detailed within U.S. EPA (2021) guides the application of strength-of-evidence judgments for environmental hazard effect within a given evidence stream and were adapted from Table 7-10 of the 2021 Draft Systematic Review Protocol (U.S. EPA, 2021).

EPA used the strength-of-evidence and uncertainties from U.S. EPA (2021) for the hazard assessment to qualitatively rank the overall confidence rating for environmental hazard (Table_Apx E-1). Confidence levels of robust, moderate, slight, or indeterminant are assigned for each evidence property that corresponds to the evidence considerations (U.S. EPA, 2021). The rank of the *Quality of the Database* consideration is based on the systematic review overall quality determination (High, Medium, or Low) for studies used to calculate the hazard threshold, and whether there are data gaps in the toxicity dataset. Another consideration in the *Quality of the Database* is the risk of bias (*i.e.,* how representative is the study to ecologically relevant endpoints). Additionally, because of the importance of the studies used for deriving hazard thresholds, the *Quality of the Database* consideration may have greater weight than the other individual considerations. The high, medium, and low systematic review overall quality determination ranks correspond to the evidence table ranks of robust, moderate, or slight, respectively. The evidence considerations are weighted based on professional judgment to obtain the overall confidence for each hazard threshold. In other words, the weights of each evidence property relative to the other properties are dependent on the specifics of the weight of scientific evidence and uncertainties that are described in the narrative and may or may not be equal. Therefore, the overall score is not necessarily a mean or defaulted to the lowest score. The confidence levels and uncertainty type examples are described below.

E.1 Confidence Levels

- Robust confidence suggests thorough understanding of the scientific evidence and uncertainties. The supporting weight of scientific evidence outweighs the uncertainties to the point where it is unlikely that the uncertainties could have a significant effect on the exposure or hazard estimate.
- Moderate confidence suggests some understanding of the scientific evidence and uncertainties. The supporting scientific evidence weighed against the uncertainties is reasonably adequate to characterize exposure or hazard estimates.
- Slight confidence is assigned when the weight of scientific evidence may not be adequate to characterize the scenario, and when the assessor is making the best scientific assessment possible in the absence of complete information. There are additional uncertainties that may need to be considered.

E.2 Types of Uncertainties

The following uncertainties may be relevant to one or more of the weight of scientific evidence considerations listed above and will be integrated into that property's rank in the evidence table:

- ***Scenario Uncertainty:*** Uncertainty regarding missing or incomplete information needed to fully define the exposure and dose.

    - The sources of scenario uncertainty include descriptive errors, aggregation errors, errors in professional judgment, and incomplete analysis.
- ***Parameter Uncertainty:*** Uncertainty regarding some parameter.
    - Sources of parameter uncertainty include measurement errors, sampling errors, variability, and use of generic or surrogate data.
- ***Model Uncertainty:*** Uncertainty regarding gaps in scientific theory required to make predictions on the basis of causal inferences.
    - Modeling assumptions may be simplified representations of reality.

Table_Apx E-1 summarizes the weight of scientific evidence and uncertainties, while increasing transparency on how EPA arrived at the overall confidence level for each exposure hazard threshold. Symbols are used to provide a visual overview of the confidence in the body of evidence, while de-emphasizing an individual ranking that may give the impression that ranks are cumulative (*e.g.,* ranks of different categories may have different weights).

Table_Apx E-1. Considerations that Inform Evaluations of the Strength of the Evidence Within an Evidence Stream (*i.e.,* Apical Endpoints, Mechanistic, or Field Studies)

Consideration	Increased Evidence Strength (of the Apical Endpoints, Mechanistic, or Field Studies Evidence)	Decreased Evidence Strength (of the Apical Endpoints, Mechanistic, or Field Studies Evidence)
The evidence considerations and criteria laid out here guide the application of strength-of-evidence judgments for an outcome or environmental hazard effect within a given evidence stream. Evidence integration or synthesis results that do not warrant an increase or decrease in evidence strength for a given consideration are considered "neutral" and are not described in this table (and, in general, are captured in the assessment-specific evidence profile tables).		
Quality of the database[a] (risk of bias)	• A large evidence base of *high*- or *medium*-quality studies increases strength. • Strength increases if relevant species are represented in a database.	• An evidence base of mostly *low*-quality studies decreases strength. • Strength also decreases if the database has data gaps for relevant species, *i.e.,* a trophic level that is not represented. • Decisions to increase strength for other considerations in this table should generally not be made if there are serious concerns for risk of bias; in other words, all the other considerations in this table are dependent upon the quality of the database.
Consistency	Similarity of findings for a given outcome (*e.g.,* of a similar magnitude, direction) across independent studies or experiments increases strength, particularly when consistency is observed across species, life stage, sex, wildlife populations, and across or within aquatic and terrestrial exposure pathways.	• Unexplained inconsistency (*i.e.,* conflicting evidence; see U.S. EPA (2005) decreases strength.) • Strength should not be decreased if discrepant findings can be reasonably explained by study confidence conclusions; variation in population or species, sex, or life stage; frequency of exposure (*e.g.,* intermittent or continuous); exposure levels (low or high); or exposure duration.
Strength (effect magnitude) and precision	• Evidence of a large magnitude effect (considered either within or across studies) can increase strength. • Effects of a concerning rarity or severity can also increase strength, even if they are of a small magnitude. • Precise results from individual studies or across the set of studies increases strength, noting that biological significance is prioritized over statistical significance. • Use of probabilistic model (*e.g.,* Web-ICE, SSD) may increase strength.	Strength may be decreased if effect sizes that are small in magnitude are concluded not to be biologically significant, or if there are only a few studies with imprecise results.
Biological gradient/dose-response	• Evidence of dose-response increases strength. • Dose-response may be demonstrated across studies or within studies and it can be dose- or duration-dependent. • Dose response may not be a monotonic dose-response (monotonicity should not necessarily be expected, *e.g.,* different outcomes may be expected at low vs. high	• A lack of dose-response when expected based on biological understanding and having a wide range of doses/exposures evaluated in the evidence base can decrease strength. • In experimental studies, strength may be decreased when effects resolve under certain experimental conditions (*e.g.,* rapid reversibility after removal of exposure).

Consideration	Increased Evidence Strength (of the Apical Endpoints, Mechanistic, or Field Studies Evidence)	Decreased Evidence Strength (of the Apical Endpoints, Mechanistic, or Field Studies Evidence)
	doses due to activation of different mechanistic pathways or induction of systemic toxicity at very high doses). • Decreases in a response after cessation of exposure (*e.g.,* return to baseline fecundity) also may increase strength by increasing certainty in a relationship between exposure and outcome (this particularly applicable to field studies).	• However, many reversible effects are of high concern. Deciding between these situations is informed by factors such as the toxicokinetics of the chemical and the conditions of exposure, see (U.S. EPA, 1998), endpoint severity, judgments regarding the potential for delayed or secondary effects, as well as the exposure context focus of the assessment (*e.g.,* addressing intermittent or short-term exposures). • In rare cases, and typically only in toxicology studies, the magnitude of effects at a given exposure level might decrease with longer exposures (*e.g.,* due to tolerance or acclimation). • Like the discussion of reversibility above, a decision about whether this decreases evidence strength depends on the exposure context focus of the assessment and other factors. • If the data are not adequate to evaluate a dose-response pattern, then strength is neither increased nor decreased.
Biological relevance	Effects observed in different populations or representative species suggesting that the effect is likely relevant to the population or representative species of interest (*e.g.,* correspondence among the taxa, life stages, and processes measured or observed and the assessment endpoint).	An effect observed only in a specific population or species without a clear analogy to the population or representative species of interest decreases strength.
Physical/chemical relevance	Correspondence between the substance tested and the substance constituting the stressor of concern.	The substance tested is an analog of the chemical of interest or a mixture of chemicals which include other chemicals besides the chemical of interest.
Environmental relevance	Correspondence between test conditions and conditions in the region of concern.	The test is conducted using conditions that would not occur in the environment.
[a] Database refers to the entire dataset of studies integrated in the environmental hazard assessment and used to inform the strength of the evidence. In this context, database does *not* refer to a computer database that stores aggregations of data records such as the ECOTOX Knowledgebase.		