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Acute Aquatic Life Screening Value for 6PPD in Freshwater

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Health and Ecological Criteria Division
Ecological Risk Assessment Branch
Washington, DC

Technical Analysis Leads:

Amanda Jarvis, Office of Water, Office of Science and Technology, Health and Ecological Criteria Division, Washington, DC

Kathryn Gallagher, Office of Water, Office of Science and Technology, Health and Ecological Criteria Division, Washington, DC

EPA Reviewers:

Will Backe, Office of Research and Development, Center for Computational Toxicology and Exposure, Great Lakes Toxicology and Ecology Division, Duluth, MN

Collin Beachum, Office of Chemical Safety and Pollution Prevention, Office of Pollution Prevention and Toxics, Existing Chemicals Risk Assessment Division, Research Triangle Park, NC

Karen Eisenreich, Office of Chemical Safety and Pollution Prevention, Office of Pollution Prevention and Toxics, Existing Chemicals Risk Assessment Division, Research, Washington, DC

Mike Elias, Office of Water, Office of Science and Technology, Health and Ecological Criteria Division, Washington, DC

Russ Erickson, Office of Research and Development, Center for Computational Toxicology and Exposure, Great Lakes Toxicology and Ecology Division, Duluth, MN

Colleen Flaherty, Office of Water, Office of Science and Technology, Health and Ecological Criteria Division, Washington, DC

Heather Goss, Office of Water, Office of Wastewater Management, Water Permits Division, Washington, DC

Dale Hoff, Office of Research and Development, Center for Computational Toxicology and Exposure, Great Lakes Toxicology and Ecology Division, Duluth, MN

Michael Hornung, Office of Research and Development, Center for Computational Toxicology and Exposure, Great Lakes Toxicology and Ecology Division, Duluth, MN

Mark Jankowski, Region 10, Laboratory Services and Applied Science Division, Seattle, WA

Sarah Kadlec, Office of Research and Development, Center for Computational Toxicology and Exposure, Great Lakes Toxicology and Ecology Division, Duluth, MN

Karen Kesler, Office of Water, Office of Science and Technology, Standards and Health Protection Division, Washington, DC

Rochelle Labiosa, Region 10, Water Division, Seattle, WA

Kevin Lott, Office of Research and Development, Center for Computational Toxicology and Exposure, Great Lakes Toxicology and Ecology Division, Duluth, MN

Dave Mount, Office of Research and Development, Center for Computational Toxicology and Exposure, Great Lakes Toxicology and Ecology Division, Duluth, MN

Ian Mundy, Office of Research and Development, Center for Computational Toxicology and Exposure, Great Lakes Toxicology and Ecology Division, Duluth, MN

Jennifer Olker, Office of Research and Development, Center for Computational Toxicology and Exposure, Great Lakes Toxicology and Ecology Division, Duluth, MN

Dan Villeneuve, Office of Research and Development, Center for Computational Toxicology and Exposure, Great Lakes Toxicology and Ecology Division, Duluth, MN

Erik Winchester, Office of Research and Development, Office of Science Advisor, Policy, and Engagement, Science Policy Division, Washington, DC

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ACRONYMS

4-DBAP	phenol, 4-[(1,3-dimethylbutyl)amino]
4-HDPA	4-hydroxydiphenylamine
6PPD	N-(1,3-Dimethylbutyl)-N′-phenyl-p-phenylenediamine
6PPD-quinone/6PPD-q	N-(1,3-Dimethylbutyl)-N′-phenyl-p-phenylenediamine-quinone
6QDI	N-(1,3-dimethylbutyl)-N'-(phenyl)-1,4-benzoquinonediimine
ACR	acute-to-chronic ratio
ASTM	American Society for Testing and Materials
AWQC	Ambient Water Quality Criteria
CAS	Chemical Abstract Services
CMC	criterion maximum concentration
CWA	Clean Water Act
DER	Data Evaluation Record
DMSO	dimethyl sulfoxide
DO	dissolved oxygen
dph	days post hatch
EC50	effect concentration for fifty percent of test organisms
ECCC	Environment and Climate Change Canada
ELS	early-life stage
EPA	United States Environmental Protection Agency
FAV	Final Acute Value
FCV	Final Chronic Value
GMAV	genus mean acute value
GMCV	genus mean chronic value
hpf	hours post fertilization
IC50	inhibitory concentration for fifty percent of test organisms
Koc	organic carbon partition coefficient
Kow	octanol/water partition coefficient
LC50	lethal concentration for fifty percent of test organisms
LOEC	lowest observed effect concentration
MATC	maximum acceptable toxicant concentration
MDR	minimum data requirement
MIC	Monsanto Industrial Chemicals
NOAA	National Oceanic and Atmospheric Administration
NOEC	no observed effect concentration
OCSPP	Office of Chemical Safety and Pollution Prevention
OECD	Organization for Economic Co-operation and Development
PPD	para-phenylenediamine
SMAV	species mean acute value
SMCV	species mean chronic value
SOP	Standard Operating Procedure
TP	Transformation products
TRWP	tire and road wear particles

TWP	tire wear particles
URMS	urban runoff mortality syndrome
UV	ultraviolet

NOTICES

This document provides information that states and authorized Tribes may consider in their water quality protection programs to protect freshwater aquatic life from the acute toxic effects of N-(1,3-Dimethylbutyl)-N′-phenyl-p-phenylenediamine (6PPD). While this document contains the United States Environmental Protection Agency's (EPA) scientific analyses regarding an acute screening value for ambient freshwater concentrations of 6PPD protective of aquatic life, including sensitive fish species, this document does not substitute for the Clean Water Act (CWA) or the EPA's regulations; nor is this document or the screening value for 6PPD it presents a regulation itself. Thus, this document does not establish or affect legal rights or obligations, or impose legally binding requirements on the EPA, states, Tribes, or the regulated community. It cannot be finally determinative of the issues addressed. This document has been approved for publication by the Office of Science and Technology, Office of Water, U.S. Environmental Protection Agency.

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FOREWORD

This document presents an acute screening value for aquatic life in ambient water based upon consideration of all available toxicity information relating to the acute effects of 6PPD on freshwater aquatic organisms. The EPA developed this document to provide information that states and authorized Tribes may consider in their water quality protection programs.

Deborah G. Nagle
Director
Office of Science and Technology

EXECUTIVE SUMMARY

This document provides the U.S. Environmental Protection Agency's (EPA) scientific basis for the development of a screening value to protect freshwater aquatic life, including sensitive fish species, from the acute effects of N-(1,3-Dimethylbutyl)-N′-phenyl-p-phenylenediamine (6PPD). This work was undertaken to fulfill a pressing need to establish protective values for 6PPD which has been found to be toxic to certain sensitive aquatic species, including sensitive fish. The EPA developed this screening value in accordance with Section 304(a)(2) of the Clean Water Act (CWA) to provide states, authorized Tribes, and other stakeholders with the best available information on the toxicity of 6PPD to aquatic organisms.

6PPD is a rubber anti-oxidant and anti-ozonant compound used in tires to protect rubber from reactions with oxygen and ozone, which can lead to degradation and cracking. The 6PPD ozonation product, 6PPD-q (N-(1,3-Dimethylbutyl)-N′-phenyl-p-phenylenediamine-quinone), was first linked to "urban stream syndrome" or "urban runoff mortality syndrome" (URMS) by Tian et al. (2021). URMS is used to describe the death of adult salmonid fish (particularly coho salmon, *Oncorhynchus kisutch*) returning to urban waterways and was first reported in Puget Sound (Washington, USA) during monitoring of urban streams between 1999 and 2001 (Scholz et al. 2011).

This screening value is distinct from the national recommended Ambient Water Quality Criteria (AWQC) that the EPA issues in accordance with the provisions of Section 304(a)(1) of the CWA for the protection of aquatic life from toxic chemicals. The limited available data for 6PPD to not fulfill the EPA's data requirements for deriving national recommended AWQC according to EPA's "*Guidelines for Deriving Numerical National Water Quality Criteria for the Protection of Aquatic Organisms and Their Uses*" (*Guidelines*; U.S.EPA 1985).

Empirical data are very limited for 6PPD and do not fulfill the minimum data requirements (MDRs) described in the EPA's *Guidelines*. In particular, the data on chronic 6PPD toxicity and on 6PPD toxicity in estuarine/marine waters is extremely limited. Additionally, the *Guidelines* recommend that toxicity data for a minimum of eight families of aquatic animals be used to fulfill MDRs in the development of aquatic life AWQC in order for criteria to reflect protection of aquatic ecosystems as a whole. However, acute toxicity data (quantitative and qualitative) for 6PPD were available for six of the eight families of aquatic animals. Further, much of the available data were developed using aquatic toxicity testing approaches that do not fully conform with the EPA's 850 Ecological Effects Test Guidelines (https://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-850-ecological-effects-test-guidelines) or other standard test guidelines, such as those of the Association for Testing and Materials (ASTM) or Organization for Coordination and Development (OECD). For example, most testing on fish was conducted over a 24-hour duration instead of the typical 96-hour test duration reflecting the researcher's consideration of the rapid onset of mortality upon exposure to 6PPD, as outlined in the document below. Additionally, when measured over the test duration toxicity tests consistently observed a loss of 6PPD, which also varied across tests and treatment groups. In order to account for the observed loss, the EPA used average exposure concentrations when available and adjusted concentrations that were measured only at the start of the test or were unmeasured, as outlined in the document below. These data limitations and deviations from standard testing methods, which are inconsistent with the EPA's *Guidelines*, made the derived toxicity values more uncertain Than national recommended AWQC. Thus, the EPA was unable to develop AWQC for this compound.

The EPA developed the 6PPD screening value for aquatic life generally following the derivation methods and calculation approach described in the EPA's *Guidelines.* The acute 6PPD screening value for aquatic life in freshwater was externally peer reviewed in the fall of 2023 by three experts in aquatic ecotoxicology. Comments from the external peer reviewers were favorable of both the screening value calculation and the data used. For complete details, please see the external peer review report and EPA's responses to peer review comments (https://www.epa.gov/wqc/acute-6ppd-aquatic-life-screening-value-freshwater). The acute screening value concentration is expected to be generally protective of 95% of freshwater species exposed to 6PPD for short durations (e.g., one hour or less). However, because only limited toxicity test data were available, the screening value is less certain than national recommended aquatic life AWQC or aquatic life benchmarks, which are both developed using more robust empirical data sets (e.g., meet most MDRs and are consistent with testing methods described in the *Guidelines* or the EPA's 850 Test Guidelines). The science and understanding of 6PPD are relatively recent (with the 6PPD ozonation product, 6PPD-q, being attributed to causing urban runoff mortality syndrome (URMS) in the past decade) and evolving, with a number of toxicity studies currently underway. As such, the EPA will continue to monitor the 6PPD literature and toxicity data to evaluate the protectiveness of this screening value.

This document provides a critical review of all aquatic ecotoxicity data identified in the EPA's literature search for 6PPD through the December 2023 quarterly update of the ECOTOXicology database (ECOTOX; https://cfpub.epa.gov/ecotox/) and provides an acute screening value for 6PPD for freshwater environments to protect sensitive aquatic life. (A separate document provides the critical review of aquatic ecotoxicity data and acute screening value for 6PPD-q).

The screening value for acute exposures to 6PPD is 8.9 µg/L (8,900 ng/L). The assessment of the available data for fish and invertebrates indicates this screening value is expected to protect the freshwater aquatic community, including sensitive fish species, from acute exposures to 6PPD. The EPA expects to update this screening value in the future as additional aquatic toxicity data become available.

1 INTRODUCTION

The EPA derived a screening value in accordance with Section 304(a)(2) of the Clean Water Act (CWS) for acute exposures to 6PPD, based upon the best available data to provide information that states and authorized Tribes may consider in their water quality programs. Section 304(a)(2) of the CWA directs the EPA to develop and publish information on the protection of aquatic life, among other things.

This screening value is distinct from national recommended Ambient Water Quality Criteria (AWQC) which are established by the EPA under Section 304(a)(1) of the CWA. Section 304(a)(1) of the CWA directs the EPA to develop and publish AWQC recommendations reflecting the latest scientific knowledge on the adverse ecological effects to aquatic life resulting from exposure to pollutants found in water. For N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylene diamine (6PPD), there were an insufficient number of toxicity tests published with data generated following standard testing procedures, through the literature review period ending in December 2023, that met the minimum data requirements (MDRs) to derive aquatic life criteria according to the EPA's "*Guidelines for Deriving Numerical Water Quality Criteria for the Protection of Aquatic Organisms and Their Uses"* (U.S.EPA 1985). In particular, the data on the chronic 6PPD toxicity and on 6PPD toxicity in estuarine/marine water are extremely limited. Further, much of the available data were developed using aquatic toxicity testing approaches that do not fully conform with the EPA's 850 Ecological Effects Test Guidelines (https://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-850-ecological-effects-test-guidelines) or other standard test guidelines, such as those of the ASTM or OECD). For example, most testing on fish was conducted over a 24-hour duration instead of the typical 96-hour test duration reflecting the researchers' consideration of the rapid onset of mortality upon exposure to 6PPD, as described below (Section 2.2.2). Additionally, when measured over

the test duration, toxicity tests consistently indicated a loss of 6PPD, which also varied across tests and treatment groups. In order to account for the observed chemical loss, the EPA used average exposure concentrations when available and adjusted concentrations that were measured only at the start of the test or were unmeasured, as outlined in the document below (Section 2.2.2.3). These data limitations and deviations from standard testing methods, which are inconsistent with the EPA's *Guidelines*, made the derived toxicity values more uncertain than national recommended AWQC.

This assessment provides a critical review of all aquatic toxicity data identified in the EPA's literature search of 6PPD through the December 2023 quarterly update of ECOTOX. It quantifies the toxicity of 6PPD to aquatic organisms, including sensitive fish species, and provides a screening value to protect aquatic life in freshwater from the acute toxic effects of 6PPD.

The EPA derived the screening value for acute exposures to 6PPD in freshwaters using the best available data to reflect the latest scientific knowledge on the toxicological effects of 6PPD to aquatic life, following the general approach outlined in the *Guidelines*, but with fewer studies and data than are typically used to develop national recommended aquatic life AWQC, resulting in greater uncertainty. The acute 6PPD screening value for aquatic life in freshwater was externally peer reviewed in the fall of 2023 by three experts in aquatic ecotoxicology. Comments from the external peer reviewers were favorable of both the calculation of the screening value and data used. For complete details, please see the external peer review report and EPA's responses to peer review comments (https://www.epa.gov/wqc/acute-6ppd-aquatic-life-screening-value-freshwater). From the limited amount of available data, the 6PPD screening value in this document is expected to be protective of sensitive organisms in freshwater aquatic

communities. The freshwater screening value is the EPA's current best estimate of the maximum concentration of 6PPD for acute (short-term) exposures, with associated frequency and duration specifications. This screening value is intended to protect freshwater aquatic species from adverse effects of acute exposure. Additional toxicity data (especially for aquatic taxa that currently have no available toxicity data, and repeated toxicity studies for previously studied taxa) are needed to fully understand the aquatic toxicity of 6PPD and to derive national recommended AWQC (that meet the MDRs as outlined in the *Guidelines* and test methods that more closely conform with EPA's 850 Ecological Effects Test Guidelines).

2 PROBLEM FORMULATION

A problem formulation provides the strategic framework for the development of a recommended water quality criteria, benchmarks, or screening values under the CWA by focusing the evaluation on the most relevant chemical properties and endpoints for consideration, to ensure the derivation of appropriate and protective aquatic life values (U.S.EPA 1998).

2.1 Fate and Transport of 6PPD in the Aquatic Environment

6PPD is an additive to vehicle tire rubber, where it functions as one of several para-phenylenediamine (PPD) additives to protect rubber from reactions with ozone and oxygen, which can lead to degradation and cracking (Baensch-Baltruschat et al. 2020; Seiwert et al. 2022). 6PPD is estimated to make up 1 to 2% (by mass) of most tires (between 10,000 to 20,000 micrograms per gram, µg/g) where it slowly migrates to the tire surface to supply a continual source of protection for the tire rubber (DTSC 2022). With usage, the total concentration of 6PPD in the tire decreases over the lifetime of the tire (DTSC 2022). By design, 6PPD is highly reactive and transforms into a number of reaction products, both known and unknown, at the surface of the tire or when released into the environment (DTSC 2022; Seiwert et al. 2022; Unice et al. 2015).

6PPD predominantly enters aquatic environments through surface runoff from roads. Tire wear particles (TWP) are generated and released as tires roll across road surfaces, particularly as vehicles brake, accelerate, and turn (Baensch-Baltruschat et al. 2020; Seiwert et al. 2022). The estimated proportion of TWP that is transferred from rainwater and surface runoff to receiving water is 13 to 45% of total emitted TWP assuming no treatment or mitigation of runoff (DTSC 2022; Wagner et al. 2018). Based on the results of eight field studies using zinc as a marker for TWP, Blok (2005) concluded that on average one third of solids emitted on roads are removed from the road system by drift while the remaining two thirds are transported by runoff. In

general, it is estimated that only a small fraction of TWP is emitted into the atmosphere while much larger portions reach soils close to roads and aquatic compartments, respectively (Baensch-Baltruschat et al. 2020). For example, Lassen et al. (2015) calculated the annual tire wear masses generated in the road network in Denmark and released into the aquatic environment considering runoff from roads and the percentage of different stormwater runoff treatment systems (e.g., roads equipped with a drainage system including runoff treatment or transport to a wastewater treatment plant). According to their results, 8 to 40% of the tire wear formed on roads in Denmark reaches surface waters. In their work to assess the environmental availability of additives in TWP, including 6PPD, Unice et al. (2015) estimated an 88% reduction from the total concentration detected in cured tread. The authors found that each lifecycle step in their conceptual model representing total environmental availability and release to water contributed incrementally to the dissipation of the parent compound or transformation product. In this study, for the parent compound 6PPD, measured transformation products included diphenylamine (DPA), 4-aminodiphenylamine (4-ADPA), 4-hydroxydiphenylamine (4-HDPA), and 4-nitrodiphenylamine (4-NDPA).

Once released into the environment, hydrolysis and photodegradation were hypothesized as major means of environmental transformation for 6PPD (OSPARCommission 2006). More recent research has confirmed environmental transformation of 6PPD produces numerous transformation products (TPs), including 6PPD-q (DTSC 2022; OSPARCommission 2006; Seiwert et al. 2022; Tian et al. 2021). Abiotic transformation of 6PPD to TPs from solid and aqueous phases during lab scale experiments resulted in the formation of 83 TPs (Seiwert et al. 2022). Of these TPs, 34 were detected in the snow from urban roads. The major load of 6PPD and its TPs in snow from urban roads was determined to be in the particulate phase,

predominantly from 6PPD and 6PPD-q (90 – 99%). The authors also found up to 13 TPs of 6PPD in the influent to a wastewater treatment plant treating combined sewer water. The proportion of 6PPD and 6PPD-q to the total signal area of all 6PPD related compounds in the dissolved phase was < 1%, outlining the increasing importance of other TPs in the dissolved phase. Finally, the estimated load of 6PPD and its TPs in municipal wastewater was greatly elevated during snowmelt and rain compared to dry weather conditions.

To date, most research on the fate and transport of 6PPD in the aquatic environment has centered on freshwater ecosystems, as detailed below. Similar information in estuarine/marine environments is currently lacking.

2.1.1 Physicochemical Properties of 6PPD

The estimated Henry's Law constant of 6PPD is 7.43 X 10^{-4} at 25°C, suggesting moderate potential to volatilize from surface waters (OSPARCommission 2006). Research summarized in the OSPARCommission (2006) report indicates no gaseous emissions of 6PPD from tires; however, it is unclear if that is due to lack of volatility from tires or rapid degradation of 6PPD once released. Although its vapor pressure appears minimal, detection of 6PPD on atmospheric particles indicates 6PPD may be present in air adsorbed to suspended particles or through resuspension of TWP or tire and road wear particles (TRWP) (Wu et al. 2020) .

Substantial data gaps remain regarding the characteristics, environmental fate, and transport of 6PPD in the aquatic environment, including the partitioning behavior into sediments and soil and biological availability to aquatic organisms. The organic carbon partition coefficient (Koc) value for 6PPD is estimated to be between 4.04 to 4.84, while the octanol-water partition coefficient (Kow) value is estimated to between 4.68 to 5.60 (DTSC 2022). This suggests a tendency for 6PPD to sorb to soils, sediments, and suspended particulate matter upon release to the environment (OSPARCommission 2006). Leaching of 6PPD through soil to groundwater is

anticipated to be unlikely (OSPARCommission 2006), although confirmation is still needed to confirm the tendency and strength with which 6PPD adheres to and remains bound to particles under environmental conditions.

The solubility of 6PPD in water appears variable and ranges from 0.5 to 2 mg/L (ECHA 2021; Hiki et al. 2021; Klöckner et al. 2020) . It is believed this variability may be due to 6PPD's high susceptibility to hydrolysis and short half-life in water (DTSC 2022).

2.1.2 Environmental Fate and Degradation

2.1.2.1 Abiotic Degradation in Controlled versus Natural Conditions

Di et al. (2022) conducted 6PPD hydrolysis experiments in buffered laboratory water at 25ºC and different pH levels (4, 7 and 9) in the dark. The hydrolysis of 6PPD (including enantiomers rac-6PPD, S-6PPD and R-6PPD) followed the first-order kinetics equation. Hydrolysis was the slowest in pH 4 water solution, followed by pH 9 water solution, and it was the fastest in pH 7 water solution. The hydrolysis half-lives in pH 4 water solution (57.3–64.1 hours) were an order of magnitude higher than those in pH 7 water solution (4.83–5.17 hours). In comparison, the hydrolysis half-lives in natural river water (hours) were within the range of those in pH 7 and pH 9 buffered water solutions, but the residual concentrations of 6PPD were higher in river water than in the pH 7 and pH 9 buffered water solutions after 48 hours.

6PPD is highly reactive with oxygen in water, with the reaction rate potentially affected by the presence of metals, pH, temperature, and sunlight (DTSC 2022; ECHA 2021; Hiki et al. 2021; OSPARCommission 2006). Depending on the environmental conditions, reported half-lives range from 3.4 hours to less than a day (ECHA 2021; OSPARCommission 2006). Kretzschmar and Neyen (1992) reported that 6PPD was stable for at least four weeks in aqueous solutions at pH 2 in the cold but degraded at neutral or basic pH within a few hours. This is consistent with the findings reported in Hiki et al. (2021) where the authors report an

experimentally-derived half-life for 6PPD of 5 hours at 23°C in dechlorinated tap water, which is increased to 8 hours at 10°C in dechlorinated tap water. Similarly, the half-life of 6PPD in well water was found to be less than one day at 24°C (Monsanto 1979). Insufficient data exist at this time to perform a formal analysis of the stability of 6PPD in natural waters, particularly in natural water from different types of aquatic systems (lotic/lentic, freshwater/saltwater), compared to synthetically prepared waters. Important factors contributing to variability in stability appear to be water pH and temperature, although additional research should be conducted to improve our understanding of other contributing factors. Nevertheless, the limited research to date indicates that abiotic degradation of 6PPD in an aqueous matrix is rapid. Accordingly, and to better reflect realistic exposures in the aquatic environment as well as the rapid resulting mortality observed in definitive laboratory toxicity tests with sensitive fish species, high quality acute tests following the exposure recommendations outlined in the EPA's 850 Ecological Effects Test Guidelines were preferred; however, tests with 24 hours (or longer) exposure to 6PPD were considered for quantitative use (see additional details regarding test duration in Section 2.2.2.4).

In the atmosphere, 6PPD has been shown to undergo indirect photodegradation via rapid reaction with hydroxyl radicals, resulting in a half-life in air on the order of one to two hours (ECHA 2021; OSPARCommission 2006). 6PPD absorbs UV-B radiation and is expected to undergo rapid direct photolysis (OSPARCommission 2006) in direct sunlight (OECD 2012). 6PPD has an estimated atmospheric half-life of 1.7 hours due to indirect photolysis with hydroxyl radicals (OECD 2004a).

2.1.2.2 Biotic Degradation

Much uncertainty exists regarding the degradation of 6PPD in the real world, as indicated previously. There is evidence to suggest the degradation of 6PPD observed in nature is likely a

result of the combination of abiotic and biotic processes (ECHA 2021; OSPARCommission 2006), but results are variable. For example, early studies indicated that 6PPD degradation was fastest in biologically-active Mississippi river water (half-life of 2.9 hours), slower in sterile river water (3.9 hours), and slowest (half-life of 6.8 hours) in sterile deionized water (OSPARCommission 2006). When evaluated in a buffered solution at either 26℃ or 50℃ and in a nutrient medium at 26℃, the hydrolysis half-lives were 14 and 5 hours for the buffered solution at 26℃ and 50℃, respectively, and 8 hours for the nutrient medium. At pH 7.0, the hydrolysis half-lives were 5.7 and 6.3 hours in light and dark deionized water, respectively, and 3.7 and 5.7 hours in light and dark well water, respectively (ToxServices 2021). In contrast, while 6PPD does not meet the OECD strict definition of readily biodegradable, when calculated based on biological oxygen demand over 28 days (OSPARCommission 2006), it does undergo rapid loss via hydrolysis, as evidenced by its 92% removal over the same period (OSPARCommission 2006), suggesting that abiotic processes are dominant. Furthermore, a 6PPD degradation study indicated comparable loss of 6PPD in river water (97%) and sterilized river water (96%) over 22 hours, indicating that biotic degradation was minimal (ECHA 2021). Additional research is needed to address these apparent confounding results and resolve existing uncertainties.

2.1.2.3 Major Degradation Products of 6PPD

Because 6PPD is so highly reactive (OECD 2004a; OSPARCommission 2006), exposure of organisms to 6PPD is likely to also include exposure to its environmental transformation products (DTSC 2022).

The major environmental degradation products for 6PPD, formed via abiotic degradation in water (e.g., hydrolysis) and/or biodegradation, are 6PPD-q (N-(1,3- dimethylbutyl)-N′-phenyl-p-phenylenediamine-quinone), 4-hydroxydiphenylamine, N-phenyl-p-benzoquinone monoimine,

phenylbenzoquinone imine, 1,3-dimethylbutylamine aniline, p-benzoquinone, and 1,3-dimethylbutylamine. Aniline is also formed to a lesser degree (Tian et al. 2021; UNEP 2006). Other known or suspected hydrolytic reaction products, including those generated via reaction with ozone, include N-(1,3-dimethylbutyl)-N'-(phenyl)-1,4-benzoquinonediimine, or 6QDI, 4-anilinophenol, p-hydroquinone, imino benzoquinone nitrone, benzoquinone dinitrone, 4-nitroso-N-phenyl-aniline and 1,3-dimethylbutanol (ECHA 2021; OECD 2004a).

In a recent study (Di et al. 2022), a total of four hydrolysis products were identified during the hydrolysis of 6PPD in purified water via N-dealkylation, mono-oxygenation and dehydrogenation. The major hydrolysis product of 6PPD was phenol, 4-[(1,3-dimethylbutyl)amino] or 4-DBAP (CAS # 63877-47-4). The formation of 4-DBAP was dependent on water solution pH, and the hydrolysis half-life was the longest in pH 4 water solution (107 hours), followed by pH 9 (5.47 hours) and pH 7 (1.23 hours) water solutions. The other primary degradation products detected in the study were 4-hydroxydiphenylamine (or 4-HDPA) and 6PPD-q. By comparison, in river water, the formation concentrations of 4-HDPA were significantly higher than other water solutions after 12 hours, and the higher residue concentrations were also observed in the 6PPD hydrolysis experiment in river water. Compared to the pH 7 and pH 9 water solutions, the relatively high concentrations of 6PPD and 4-HDPA in river water indicated that the harmful effects of 6PPD and its degradants might be noteworthy in natural water. Furthermore, 6PPD-q was detected in pH 4 water solutions with 6PPD, and its concentrations exceeded toxicity thresholds for sensitive freshwater species. Hu et al. (2022) reported that under ozone exposure, primary molar yields of 9.7% and 0.95% occurred for 6PPD-q formation from pure 6PPD and from 6PPD within TWP, respectively, suggesting that a substantial mass fraction of 6PPD ultimately reacted to form 6PPD-q. It is worth noting here that

in Di et al. (2022), the authors observed that the formation rate of enantiomer S-6PPD-q from S-6PPD was 1.77 times faster than in enantiomer R-6PPD-q from R-6PPD, indicating different environmental behaviors for the various TPs/mixtures, which may affect the accuracy of risk assessments.

While primary transformation products have been relatively well characterized and are produced rapidly, secondary transformation products are less well understood (ECHA 2021) and are not always recovered in degradation experiments (OSPARCommission 2006). This suggests that primary transformation products, in particular 3-hydroxydiphenyl-amine and benzoquinone-monoimine, are likely more stable than the parent 6PPD (ECHA 2021). At this point in time, decoupling the toxicological effects of 6PPD from those of its transformation products is difficult.

2.2 Measurement Endpoints

2.2.1 Overview of Toxicity Data Requirements

The *Guidelines* (U.S.EPA 1985) indicate that acute toxicity test data from a minimum of eight diverse taxonomic groups are needed to ensure protection of the aquatic community from short term exposures:

a. fish in the family Salmonidae in the class Osteichthyes
b. a second family of fish in the class Osteichthyes, preferably a commercially or recreationally important warmwater species (e.g., bluegill, channel catfish)
c. a third family in the phylum Chordata (may be in the class Osteichthyes or may be an amphibian)
d. a planktonic crustacean (e.g., cladoceran, copepod)
e. a benthic crustacean (e.g., ostracod, isopod, amphipod, crayfish)
f. an insect (e.g., mayfly, dragonfly, damselfly, stonefly, caddisfly, mosquito, midge)
g. a family in a phylum other than Arthropoda or Chordata (e.g., Rotifera, Annelida, Mollusca)
h. a family in any order of insect or any phylum not already represented

Additionally, to ensure the protection of various animal components of the aquatic ecosystem from long term exposures, chronic toxicity test data are recommended from the same eight diverse taxonomic groups that are recommended for acute criteria. If data for the eight diverse taxonomic groups are not available to support the chronic criterion derivation using a genus distribution approach, the chronic criterion may be derived using an acute-to-chronic ratio (ACR) approach.

There were only two chronic toxicity studies for 6PPD (**Error! Reference source not found.** and **Error! Reference source not found.**). Therefore, a freshwater chronic screening value could not be derived at this time. However, given the short half-life of 6PPD and the rapid mortality in tests with several species, acute toxicity is expected to be a more important driver for aquatic risk than chronic toxicity.

The *Guidelines* document also specifies that quantitative toxicity test data be available for at least one freshwater alga or vascular plant. If plants are among the most sensitive aquatic organisms, toxicity test data from a plant in another phylum should also be considered. A 6PPD literature search was conducted through the December 2023 ECOTOX update for freshwater alga or vascular plants; however, there were no available toxicity data for these taxa. Therefore, the EPA was unable to determine the relative toxicity of 6PPD to aquatic plants (based on the latest literature search and review completed December 2023). Therefore, this screening value was derived without the use of aquatic plant data.

2.2.2 Data Acquisition and Measure of 6PPD Exposure Concentrations

All acute freshwater studies with 6PPD-only exposures (no exposures to mixtures) through the December 2023 ECOTOX update were reviewed for data quality for possible inclusion in the derivation of the screening value. Tests determined to be of sufficient quality were used quantitatively for calculating the 6PPD screening value. Studies not included in the

numeric screening value derivation were either considered qualitatively as supporting information, if determined to be of sufficient quality, or were rejected from further consideration. These data are described in the Effects Characterization (Section 4).

Published toxicity data identified as meeting quality standards and included in the ECOTOXicology database (ECOTOX; https://cfpub.epa.gov/ecotox/) were considered for use in deriving the screening value. ECOTOX is a source of high-quality toxicity data for aquatic life, terrestrial plants, and wildlife. The ECOTOX database was created and is maintained by the EPA, Office of Research and Development, Center for Computational Toxicology and Exposure. The ECOTOX search process typically begins with a comprehensive chemical-specific literature search of the open literature conducted according to ECOTOX Standard Operating Procedures (SOPs; Elonen 2020). Consistent with the objective of being comprehensive, the initial searches often encompass multiple chemical terms, synonyms, degradates and verified Chemical Abstracts Service (CAS) numbers. After developing the literature search strategy and completing the initial search, ECOTOX curators then identify potentially applicable studies based on title and abstract, acquire potentially applicable studies, and utilize the applicability criteria for inclusion in ECOTOX (U.S.EPA 2022).

Following inclusion in the ECOTOX database, toxicity studies were further evaluated by the EPA Office of Water (OW). All studies were evaluated for data quality as described in the *Guidelines*, the EPA's Office of Chemical Safety and Pollution Prevention (OCSPP)'s Ecological Effects Test Guidelines (U.S.EPA 2016b), and the EPA OW's internal data quality SOP, which is consistent with OCSPP's data quality review approach (U.S.EPA 2018). OW completed a Data Evaluation Record (DER) for each of the 6PPD studies identified in ECOTOX.

This in-depth review ensured the studies used to derive the screening value resulted in a robust, transparent, and scientifically-defensible outcome.

Due to the relatively limited dataset for 6PPD, the EPA had to make several adaptations to the traditional (*Guidelines*) approach in order to develop the screening value, as described below. The EPA determined that despite the deviations from typical acute study designs, the agency would proceed with generating an acute screening value because of the importance of developing protective values for this highly toxic chemical for states and Tribes to consider in their water quality protection programs.

2.2.2.1 Use of Non-Native Taxa

The EPA typically develops national recommended aquatic life criteria, benchmarks, and screening values using toxicity data from North American resident species (as per the *Guidelines*). Due to the limited aquatic life data landscape for 6PPD, the EPA has relaxed this recommendation and instead has developed the screening value using all available aquatic toxicity data meeting data quality objectives, regardless of where the species resides globally. In this context, species not resident to North American serve as taxonomically-related surrogate test organisms for the thousands of untested North American resident species.

2.2.2.2 Use of Nominal Concentrations

A number of 6PPD toxicity tests reported only nominal, or unmeasured, 6PPD concentrations. Given the limited availability of 6PPD toxicity data for aquatic life, reported nominal concentrations were used for several studies without reported measured 6PPD concentrations, in addition to studies reporting measured 6PPD concentrations. This approach is consistent with the *Guidelines*, which states that acute toxicity data from all measured flow-through tests would be used to calculate species mean acute values (SMAV), unless data from a measured flow-through test were unavailable, in which case the acute criterion would be

calculated as the geometric mean of all the available acute values (i.e., results of unmeasured flow-through tests and results of measured and unmeasured static and renewal tests). Therefore, the EPA used both measured and unmeasured toxicity tests in the development of this acute 6PPD screening value, which is consistent with the *Guidelines*.

2.2.2.3 Use of Averaged Test Concentrations over Exposure Duration to Account for 6PPD Loss over the Duration of the Tests

The 6PPD toxicity studies in the current literature consist of a mixture of measured tests with concentrations measured: (1) at the beginning of the tests, and (2) with measurements at both the beginning and end of the tests, which reported averaged concentrations. When available, the EPA used the averaged concentrations in the calculation of the screening value. In instances where only nominal concentrations or measured concentrations at the initiation of the exposure were reported, the EPA adjusted the exposure concentration to account for expected loss during testing. This adjustment was based on studies that measured 6PPD at different time points throughout the experiment. Specifically, Prosser et al. (2017a) reported a 75 to 90% loss of 6PPD over 96 hours while Prosser et al. (2017b) reported a loss of 6PPD of 70 to 94%. These ranges were averaged for each study (e.g., average of 82.5% for (Prosser et al. 2017a)) and the geometric mean of the averages were taken and divided by a factor of two to represent the average concentration over the exposure duration considering the initial and final concentrations, resulting in an approximately 40% loss across studies and treatments, Therefore, for studies with nominal or initial measured concentrations, the LC_{50} value was reduced by 40% to account for the expected loss and to make the concentrations comparable with averaged measured concentrations. These specific adjustments are noted in the individual study summaries.

2.2.2.4 Test Exposure Duration

The EPA's 850 Ecological Effects Test Guidelines specify that acute toxicity tests on fish should have at least 72 hours of exposure and recommend 96-hour exposures (U.S.EPA 2016b). However, several studies for 6PPD conducted tests with 24 hours of exposure, citing that the shortened exposure duration represented realistic exposures (e.g., via stormwater/runoff events) in the aquatic environment, and the rapid mortality in definitive laboratory toxicity tests with several of the most sensitive fish species. Given the typical exposure durations (i.e., a few hours) associated with the onset of acute toxicity in the aquatic environment and the expected speed at which 6PPD degrades in ambient waters that are well oxygenated (see Section 2.1.2), high quality acute tests following the exposure recommendations outlined in the EPA's 850 Ecological Effects Test Guidelines were preferred; however, tests with 24 hours (or longer) exposure to 6PPD were considered for quantitative use.

2.2.2.5 Biomass Loading

Several studies consisted of study designs that exceeded the EPA's Ecological Effects Test Guidelines for biomass loading in fish toxicity studies (generally of 0.8 g/L in static tests for most fish species; U.S.EPA 2016b). Nevertheless, if other study parameters were consistent with test quality guidelines and the study authors reported that the test organisms did not appear to be stressed and test conditions were acceptable (i.e., the animals exhibited high control survival and were exposed to acceptable levels of dissolved oxygen and ammonia), then the test was considered for quantitative use in the derivation of this acute screening value, due to the paucity of other data for this toxicant of high concern.

2.2.2.6 Chemical Purity

A few of the toxicity studies (Japan Ministry of the Environment 2019; Monsanto Co. 1979 and 1984) did not report chemical purity of the test compound. The EPA's 850 Ecological

Effects Test Guidelines (U.S.EPA 2016a) state that studies should indicate the exact nature and source of the chemical being tested, including the grade and purity, and that substances less than 80% pure are typically deemed unacceptable. Given the relativity limited data available for 6PPD, in the few cases where an individual toxicity test did not report the chemical purity, other information was taken into account when determining the use of the test in the derivation of the screening value. Specifically, the source of the test compound, the test method or guideline followed (e.g., OECD or the EPA's 850 Ecological Effects Test Guidelines), and the use of these tests in OECD's 6PPD assessment (OECD 2004a) when determining if a test without reported chemical purity could be used. These specific instances are noted and justifications in the use classifications for these tests are documents in individual study summaries below.

2.2.3 Measures of Effect

The acute measures of effect on aquatic organisms are the median lethal concentration (LC_{50}), effect concentration (EC_{50}), or inhibitory concentration (IC_{50}) estimated to produce a specific effect in 50 percent of the test organisms (Table 2-1). LC_{50} is the concentration of a chemical that is estimated to kill (or immobilize) 50 percent of the test organisms. EC_{50} is the concentration of a chemical that is estimated to produce a specific effect in 50 percent of the test organisms. The IC_{50} is the concentration of a chemical that is estimated to inhibit some biological process (e.g., enzyme activity associated with an apical endpoint such as mortality) in 50 percent of the test organisms.

Consistent with past practice (U.S.EPA 2013), a decision rule was also applied to the 6PPD toxicity data when an author-reported No Observed Effect Concentration (NOEC) or Lowest Observed Effect Concentration (LOEC) was used. The decision rule was not to use "greater than" values for concentrations of low magnitude or "less than" values for concentrations of high magnitude because they did not provide a definitive toxicity value.

Conversely, if data from studies with only low concentrations indicated a significant effect (suggesting the test material was highly toxic) or studies with high concentrations only found an incomplete response for an endpoint (indicating low toxicity of the test material), those data did significantly enhance the understanding of 6PPD toxicity. Thus, the decision rule was applied as follows: "greater than" (>) high toxicity values and "less than" (<) low toxicity values were included (e.g., SMAVs), but "greater than" (>) low toxicity values and "less than" (<) high toxicity values were not used in data calculations (U.S.EPA 2013). Data that met the quality objectives and test requirements were utilized quantitatively in deriving this 6PPD acute screening value and are presented in Section 3.1.1 and Appendix A.

Table 2-1. Summary of Assessment Endpoints and Measures of Effect Used in the Derivation of Aquatic Life Effect Values.

Assessment Endpoints for the Aquatic Community	Measures of Effect
Aquatic Life: Acute: Survival Chronic: Survival, growth, and reproduction of freshwater and estuarine/marine aquatic life (i.e., fish, amphibians, aquatic invertebrates)	For effects from acute exposure: 1. LC_{50}, EC_{50}, or IC_{50} concentrations in water 2. NOEC and LOEC concentrations in water For effects from chronic exposure: 1. NOEC and LOEC concentrations in water

LC_{50} = 50% Lethal Concentration
EC_{50} = 50% Effect Concentration
IC_{50} = 50% Inhibitory Concentration
NOEC = No-observed-effect-concentration
LOEC = Lowest-observed-effect-concentration

For the purpose of this document, chronic and other measures of effect are of secondary focus because the number of acceptable chronic studies of freshwater animals and aquatic plants, as well as estuarine and marine animals and plants, is non-existent. Because insufficient data exist to calculate a chronic screening value in freshwater and acute or chronic screening values in estuarine/marine waters, these data are only provided to document that the EPA reviewed and considered all available and relevant toxicity test data through the December 2023 quarterly

update of ECOTOX. The EPA expects to update the acute screening value provided herein and develop additional screening values and/or criteria in the future as new aquatic toxicity data become available.

2.3 Analysis Plan

2.3.1 Derivation of an Acute Freshwater Aquatic Life Screening Value

During the development of this screening value for acute exposures of 6PPD in freshwater, the EPA reviewed and considered all relevant acute toxicity test data through the December 2023 quarterly update of ECOTOX. Information available for all relevant species and genera were reviewed to identify: 1) data from acceptable tests that meet data quality standards; and 2) whether the acceptable data meet the MDRs as outlined in the EPA's *Guidelines* (U.S.EPA 1985). The MDRs described in Section 2.2.1 were not met for acute freshwater criteria derivation. Acceptable studies of aquatic algae and vascular plants were also not available, nor were there any acceptable acute and chronic studies of estuarine and marine animals and plants. Consequently, national recommended 304(a) AWQC for the protection of aquatic life could not be derived for 6PPD at this time. However, the EPA was able derive an acute screening value for 6PPD in freshwater. The EPA derived an acute screening value generally following the *Guidelines* method, except for a handful of adaptations noted above (Section 2.2.2).

This assessment quantifies the toxicity of 6PPD to aquatic organisms to protect aquatic life in freshwater from acute toxic effects of 6PPD. The 6PPD screening value is expected to be protective of most sensitive aquatic organisms in the community. However, this screening value for 6PPD is based on more limited empirical data, including some data developed using methods not adhering to common toxicity testing guidelines (e.g., the EPA's 850 Ecological Effects Test Guidelines), than an aquatic life criterion would be and therefore has greater inherent

uncertainty. The EPA intends to update this screening value as more data become available on the toxicity of 6PPD.

3 EFFECTS ANALYSIS FOR AQUATIC LIFE

All available studies relating to the acute and chronic toxicological effects of 6PPD on aquatic life were considered. Data for possible inclusion were obtained from published literature reporting acute and chronic exposures of 6PPD to freshwater and estuarine and marine aquatic life that were associated with mortality, survival, growth, and reproduction. As noted above, acceptable chronic studies of freshwater animals were limited, and acceptable studies on aquatic algae and vascular plants, as well as on estuarine and marine animals and plants, currently do not exist. Therefore, EPA was only able to derive an acute screening value for 6PPD in freshwater. Acute data meeting quality objectives were utilized quantitatively in deriving the screening value for acute exposures to 6PPD in freshwater and are presented in Section 3.1.1 and Appendix A. Chronic data meeting quality objectives are presented in Section 3.1.3 and Appendix C. No quantitatively acceptable acute or chronic estuarine/marine data were available at the time of the literature review (completed in December 2023).

3.1 Summary of 6PPD Toxicity Studies Considered Quantitatively to Derive the Aquatic Life Screening Value

Acute 6PPD toxicity data considered in deriving the acute aquatic life screening value were available for nine freshwater species, representing eight genera and six families in three phyla, and no estuarine/marine species (Table 3-1). There were limited quantitatively acceptable chronic 6PPD toxicity data for freshwater species, and there were no quantitatively acceptable chronic toxicity data for estuarine/marine species at the time of the literature review (completed in December 2023). The following study summaries present the key acute freshwater toxicity data with effect values that were used quantitatively to derive the acute freshwater screening value to protect aquatic life. Abbreviated study summaries are provided below for the four most sensitive acute freshwater taxa with effect values that were used quantitatively to derive the acute

screening value. Full study summaries for these and all other studies with effect values used quantitatively to derive the screening value are presented in Appendix A. The abbreviated study summaries below are presented in order of taxonomic sensitivity to 6PPD (Table 3-2) from most to least. Acute values are presented as reported by the study authors for each individual study, unless stated otherwise.

Table 3-1. Summary Table of Minimum Data Requirements per the *Guidelines* Reflecting the Number of Acute and Chronic Genus and Species Level Mean Values in the Freshwater and Saltwater Toxicity Datasets for 6PPD.

	Freshwater			
MDR[a]	**GMAV**	**SMAV**	**GMCV**	**SMCV**
Family Salmonidae in the class Osteichthyes	1	1	0	0
Second family in the class Osteichthyes, preferably a commercially or recreationally important warmwater species	3	3	1	1
Third family in the phylum Chordata (may be in the class Osteichthyes or may be an amphibian, etc.)	1	1	1	1
Planktonic Crustacean	1	1	0	0
Benthic Crustacean	1	1	0	0
Insect	0	0	0	0
Family in a phylum other than Arthropoda or Chordata (e.g., Rotifera, Annelida, or Mollusca)	1	2	0	0
Family in any order of insect or any phylum not already represented	0	0	0	0
Total	**8**	**9**	**2**	**2**
	Saltwater			
MDR[a]	**GMAV**	**SMAV**	**GMCV**	**SMCV**
Family in the phylum Chordata	0	0	0	0
Family in the phylum Chordata	0	0	0	0
Either the Mysidae or Penaeidae family	0	0	0	0
Family in a phylum other than Arthropoda or Chordata	0	0	0	0
Family in a phylum other than Chordata	0	0	0	0
Family in a phylum other than Chordata	0	0	0	0
Family in a phylum other than Chordata	0	0	0	0
Any other family	0	0	0	0
Total	**0**	**0**	**0**	**0**

a The *Guidelines* require that data from a minimum of eight families are needed to calculate a freshwater or estuarine/marine criterion. Insufficient data exist to fulfill all eight of the taxonomic MDR groups. Consequently, the EPA cannot derive a freshwater or estuarine/marine acute criterion for 6PPD, based on the *Guidelines* approach. However, the EPA has developed a screening value for acute exposures to 6PPD in freshwater through use of all quantitatively- and qualitatively-acceptable acute toxicity data.

3.1.1 Summary of Acute 6PPD Toxicity Studies Used to Derive the Freshwater Aquatic Life Screening Value

The following abbreviated study summaries present the key (four most sensitive) acute freshwater toxicity data with effect values that were used quantitatively to derive the acute screening value. Full study summaries are presented in AppendixAppendix A. Summaries are presented in order of taxonomic sensitivity to 6PPD (Table 3-2) based on sensitivity at the genus level. Acute values are presented as reported by the study authors for each individual study, unless stated otherwise. Per above, the EPA carefully reviewed other qualitatively acceptable data to increase the understanding of 6PPD toxicity and to determine with MDRs could be met with those data. Study summaries of these qualitative test data used to support derivation of the screening value are summarized in Section 4.2.

Table 3-2. Freshwater Acute 6PPD Studies Considered for Quantitative Use.
Ranked by LC_{50} and Genus Mean Acute Values (lowest to highest). Values used in the SMAV/GMAV calculation are bolded.

Rank	Genus	Species	Method[a]	Biomass Loading (g/L)[b]	Author – Reported LC_{50} (µg/L)	EPA Calculated / Adjusted EC_{50}/LC_{50} (µg/L)	SMAV (µg/L)	GMAV (µg/L)	Comment	Reference
1	*Oryzias*	Medaka, *Oryzias latipes*	S, M	Not Stated – (Assumed to be in accordance with OECD Guidelines)	**28**	-	28	28	OECD (2004) accepted this value in its analysis, however, only a table noting the study followed OECD guidance was available in the OECD document. The study itself was not available for the EPA to review for data quality.	Japan Ministry of the Environment (2019)
2	*Gobiocypris*	Rare minnow, *Gobiocypris rarus*	R, M	0.96	162	**94.94**	94.94	94.94	Missing exposure details in the paper. The EPA reached out to the study authors and is awaiting response	Di et al. (2022)
3	*Oncorhynchus*	Coho salmon (juvenile, 0-2 yr), *Oncorhynchus kisutch*	S, U	0.347 – 7.47	251	**143.7**	143.7	143.7	Duration too short (24 hours)[c]; limited test details. Despite the high biomass loading[b] there was 0% mortality in controls and D.O. saturation > 60% during the test (ammonia not reported)	Tian et al. (2021)
4	*Hyalella*	Amphipod (juvenile, 7-11 d), *Hyalella azteca*	S, M	N/A	250	**159.7**	159.7	159.7	Acute water only test is quantitative; chronic sediment exposure is considered for qualitative use (see Effects Characterization). Test concentrations were measured twice over the course of the experiment and separate LC_{50} values were calculated in the paper for test initiation and conclusion. The EPA reached out to the study authors to obtain treatment level data and is awaiting response	Prosser et al. (2017a)
5	*Daphnia*	Cladoceran (<24 hr), *Daphnia magna*	S, U	N/A	510	**306.0**	213.4	213.4		Monsanto Co. (1984)

Rank	Genus	Species	Method[a]	Biomass Loading (g/L)[b]	Author – Reported LC_{50} (µg/L)	EPA Calculated / Adjusted EC_{50}/LC_{50} (µg/L)	SMAV (µg/L)	GMAV (µg/L)	Comment	Reference
		Cladoceran (Age not stated) *Daphnia magna*	S, M	N/A	**230**	-			Value provided in a table with a footnote that OECD guidance was followed. EPA was unable to judge against data quality objectives; however, since Japan is a member of OECD, EPA assumed that test quality guidelines were met	Japan Ministry of the Environment (2019)
		Cladoceran (<24 hr), *Daphnia magna*	S, M	N/A	**< 138**	-			Only one exposure concentration resulting in no definitive effect value, a less than low value[d]	Hiki et al. (2021)
6	*Pimephales*	Fathead minnow, *Pimephales promelas*	F, M	1.3	450	**270.0**	270.0	270.0		Monsanto Co. (1979)
7	*Lampsilis*	Wavy-rayed lampmussel (glochidia), *Lampsilis fasciola*	S, M	N/A	260.0	**156.0**	156.0	299.0		Prosser et al. (2017b)
		Fatmucket (glochidia), *Lampsilis siliquoidea*	S, M	N/A	955.0	**573.0**	573.0			
8	*Danio*	Zebrafish (embryo), *Danio rerio*	R, U	N/A	442.6	**265.6**	342.7	342.7	*D. rerio* is a common aquatic toxicity test species that serves as a surrogate for untested fish species residing in North America. Zebrafish embryo test biomass loading was not an issue	Varshney et al. (2022)
		Zebrafish (embryo, 2 hpf), *Danio rerio*	R, U	N/A	737	**442.2**			*D. rerio* is a common aquatic toxicity test species that serves as a surrogate for untested fish species residing in North America	Fang et al. (2023)

[a] S=static, R=renewal, F=flow-through, U=unmeasured, M=measured

[b] The EPA's 2016 Ecological Effects Test Guidelines (OCSPP 850.1075) for Freshwater and Saltwater Fish Acute Toxicity Tests recommend that biomass loading should be ≤ 0.8 g wet weight (ww) per liter (g/L) in static or static-renewal tests and ≤ 0.5 g/L per 24 hours and < 5 g/L at any time in flow-through tests.

[c] The EPA's 2016 Ecological Effects Test Guidelines (OCSPP 850.1075) for Freshwater Fish Acute Toxicity Tests state that the test duration should be 96 hours.

[d] Consistent with past practice, a decision rule was also applied to the 6PPD toxicity data as follows: “greater than” (>) high toxicity values and “less than” (<) low toxicity values were included (U.S.EPA 2013).

3.1.1.1 Most acutely sensitive genus: *Oryzias* (Medaka)

Ministry of the Environment, Japan (2019) performed a 96-hour static, measured acute test (based on study details provided in OECD (2004a)) of 6PPD with the Japanese medaka, *Oryzias latipes*. This is a common aquatic toxicity test species that serves as a surrogate for untested fish species residing in North America. The toxicity test method used followed OECD TG 203 (Fish Acute Toxicity Test) (OECD 1992). No details were provided with regards to source of the fish, preparation of test solutions, and exposure conditions. Adequate control survival and other test acceptability requirements were assumed per OECD test guidelines. The author-reported 96-hour LC_{50} for the test was 28 µg/L. It is unclear when the test concentration measurements were taken (assumed to be average concentrations). Therefore, the author-reported value was used. The value was considered acceptable for quantitative use despite some missing exposure and test details given that the test was conducted by the Ministry of the Environment in Japan and followed OECD test guidelines and was accepted for use by the OECD (2004a). Missing data included a lack of information on the chemical purity of 6PPD used in the toxicity test. The source of 6PPD used was assumed to be of high purity (> 98%) since the test followed OECD test guidelines per OECD's 6PPD assessment (OECD 2004a). Further, it appears the source of the 6PPD was Bayer AG and OECD (2004a) states "*In Germany 6PPD is manufactured in an industrial scale only at the Bayer AG Brunsbüttel plant. In a continuously working closed system 4-aminodiphenylamine is reacted with an excess of methyl isobutyl ketone (MIBK) to a Schiff´s base. This base is then hydrogenated catalytically. The excess of MIBK is separated off. The hydrogenation by-products are purged with steam. Impurities are removed by distillation under reduced pressure yielding 6PPD with a purity of > 98 %.*"

3.1.1.1.1 Oryzias GMAV calculation

As no other quantitative toxicity values were available for this species or genus, the author-reported LC_{50} of 28 µg 6PPD/L served directly as the SMAV and GMAV.

3.1.1.2 Second most acutely sensitive genus: *Gobiocypris* (rare minnow)

Di et al. (2022) performed a 96-hour static-renewal, measured acute test of 6PPD (≥98% purity) with the rare minnow, *Gobiocypris rarus*. This species is not a North American resident species but is a common aquatic toxicity test species that serves as a surrogate for untested fish species residing in North America. The acute test followed OECD 203 methodology (OECD 1992). Eight fish (0.18 g) were added to each test vessel containing 1.5 L of solution, and test solutions were renewed every 12 hours over the course of the experiment. Each test treatment was replicated three times. Solvent controls were maintained in dechlorinated tap water and acetonitrile (solvent volume not provided) and there were five test treatments (112, 135, 162, 194 and 233 µg/L measured 6PPD). The author-reported 96-hour LC_{50} was 162 µg/L 6PPD. The EPA calculated an LC_{50} of 158.23 µg/L based on measured concentrations provided in the paper, however it is unclear when these measurements were taken during the exposure. It was assumed measured concentrations were initial concentrations. The EPA-calculated LC_{50} value was adjusted to lower the value by 40% in order to account for loss of 6PPD over experiment. The adjusted EPA-calculated LC_{50} was 94.94 µg/L 6PPD, which was acceptable for quantitative use.

3.1.1.2.1 Gobiocypris GMAV calculation

As no other quantitative toxicity values were available for this species or genus, the adjusted EPA-calculated LC_{50} of 94.94 µg 6PPD/L served directly as the SMAV and GMAV.

3.1.1.3 Third most acutely sensitive genus: *Oncorhynchus* (salmon)

Tian et al. (2021) performed a 24-hour static, unmeasured test of 6PPD (95% purity) with juvenile (0-2 yr) coho salmon, *Oncorhynchus kisutch*. Dilution water was dechlorinated

municipal water treated by reverse osmosis and reconstituted with buffered Instant Ocean salts. Eight fish per concentration were exposed in 30 L of test solution, with a 10-concentration dilution series. A solvent control (material and amount by volume not provided) and a positive control (250 mg/L tire wear particle leachate) were additionally included. The exposure was repeated twice. The author-reported 24-hour LC_{50} was calculated as 251 µg/L based on nominal concentrations of 6PPD. The EPA calculated an LC_{50} of 239.56 µg/L based on the concentration-response (C-R) data reported in the publication. Since test concentrations were unmeasured, the EPA-calculated LC_{50} value was adjusted to lower the value by 40% in order to account for loss of 6PPD over the experiment. The adjusted EPA-calculated LC_{50} was 143.7 µg/L 6PPD and is acceptable for quantitative use despite the short duration (24 hours opposed to 96 hours), as this duration represented realistic exposures in the aquatic environment.

3.1.1.3.1 Oncorhynchus GMAV calculation

As no other quantitative toxicity values were available for this species or genus, the adjusted EPA-calculated LC_{50} of 143.7 µg 6PPD/L served directly as the SMAV and GMAV.

3.1.1.4 Fourth most acutely sensitive genus: *Hyalella* (amphipod)

Prosser et al. (2017a) performed a 96-hour static, measured acute test of 6PPD (>98% purity) with the amphipod, *Hyalella azteca*. Dechlorinated City of Burlington tap water was spiked with a concentrated solution of 6PPD in acetone. Fifteen juvenile amphipods (7-11 days old) were added to 250 mL glass beakers with 200 mL of test solution and a piece of cotton gauze. Beakers were gently aerated throughout the exposure. Treatments included a control and solvent control (<0.1% by volume) and nominal test concentrations of 125, 250, 500, 1,000 and 2,000 µg/L 6PPD. Each treatment was replicated three times. Measured concentrations averaged 22% lower than nominal concentrations overall at test initiation. Only three of the ninety amphipods died in the negative (1 out of 45) and solvent (2 out of 45) controls. Mean measured

6PPD concentrations at test initiation were 15-31% less than nominal concentrations and 75-90% less at test termination. The author-reported 96-hour LC_{50} was 250 µg/L 6PPD, based on initial concentrations. The EPA curve fit the C-R data to calculate a LC_{50} value based on average concentrations instead of initial concentrations. The EPA-calculated LC_{50} was 159.7 µg/L 6PPD, which was acceptable for quantitative use.

3.1.1.4.1 Hyalella GMAV calculation

As no other quantitative toxicity values were available for this species or genus, the EPA-calculated LC_{50} of 159.7 µg 6PPD/L served directly as the SMAV and GMAV.

3.1.2 Summary of Quantitatively Acceptable Acute 6PPD Toxicity Studies for Estuarine/Marine Species

There were no quantitatively acceptable data for acute 6PPD toxicity for estuarine/marine species at the time of the literature review (completed in December 2023).

3.1.3 Summary of Quantitatively Acceptable Chronic 6PPD Toxicity Studies for Freshwater Species

The chronic freshwater dataset contains two genera representing only two taxonomic MDR groups. Quantitatively acceptable data for chronic 6PPD toxicity were available for two freshwater fish species, representing two genera and two families. Study summaries are presented below in order of genus sensitivity to 6PPD. Given the limited data currently available for 6PPD a chronic screening value for aquatic life in freshwater was not derived at this time. However, given the rapid degradation of 6PPD as well as the rapid onset of mortality observed in tests across several species, acute toxicity is expected to be a more important driver for aquatic risk compared to chronic toxicity.

3.1.3.1 Most chronically sensitive genus: *Oryzias* (Medaka)

Ministry of the Environment, Japan (2019) performed a static, unmeasured early-life stage (ELS) test of unknown duration with 6PPD on the Japanese medaka, *Oryzias latipes*. *O.*

latipes is a common aquatic toxicity test species that serves as a surrogate for untested fish species residing in North America. The toxicity test method used followed OECD TG 210 (Fish Early Life Stage Toxicity Test). No details were provided with regards to source of fish, preparation of test solutions, or exposure conditions. Adequate control survival and other test acceptability requirements were assumed per OECD test guidelines. In addition, the source of 6PPD used was assumed to be of high purity (> 98%) for reasons described above (Section 3.1.1.1). The reported NOEC and LOEC for the ELS test were 3.7 and 11 µg/L (MATC = 6.380 µg/L). The MATC was considered acceptable for quantitative consideration in development of a screening value (had data on other taxa been sufficient to develop a chronic screening value).

3.1.3.2 Second most chronically sensitive genus: *Pimephales* (fathead minnow)

Monsanto Co. (1979) performed a 28-day flow-through, measured chronic test of 6PPD [purity not reported; assumed to be high purity (> 98%) since the test was part of OECD's 6PPD assessment (OECD 2004a)] with juvenile fathead minnow, *Pimephales promelas*. Stock solutions were prepared in nanograde acetone. Diluent was aerated well water. Details about the use of a solvent control were not provided. A Mount and Brungs style proportional diluter system was used to deliver five nominal concentrations of 6PPD: 0.066, 0.12, 0.23, 0.45, and 1.0 mg/L and control (well water) to each of the six 30 L glass test aquaria after test solutions had been flowing through the aquaria for 24 hours. Each aquaria held 30 fathead minnows (1.3 g, 40.1 mm) and received control water or test solution at a rate of 300 mL/minute. The mean measured concentrations of 6PPD were determined on days 0, 1, 5, 10, 14, 21, and 28 and were 0.024, 0.034, 0.089, 0.26 and 0.92 mg/L. Measured concentrations were 92, 58, 39, 28 and 36% of the nominal concentrations progressing from highest to lowest concentration. No mortality was observed after 24 hours of exposure across all treatments. Mortality in 6PPD treatments increased as time progressed, with no mortality observed in the control and nominal 0.066 mg/L

6PPD treatment at test termination. The 28-day LC_{50} based on nominal concentrations was calculated as 0.150 mg/L, or 150 µg/L 6PPD. The test result was considered acceptable for quantitative consideration in development of a screening value (had other taxa data been sufficient to develop a chronic screening value) despite the unreported chemical purity for the test compound 6PPD as this test was used in OECD's 6PPD assessment (OECD 2004a).

3.1.4 Summary of Quantitatively Acceptable Chronic 6PPD Toxicity Studies for Estuarine/Marine Species

There are no quantitatively acceptable empirical data for chronic 6PPD toxicity for estuarine/marine species at the time of the literature review (completed in December 2023).

3.2 Derivation of Aquatic Life Screening Values for 6PPD

3.2.1 Derivation of Screening Value for Freshwater

There are insufficient data to derive a national recommended freshwater AWQC for 6PPD. The acute data set for 6PPD contains eight genera representing six taxonomic MDR groups (Table 3-3). Further, much of the available data were developed using aquatic toxicity testing approaches that do not fully conform with the EPA's 850 Ecological Effects Test Guidelines or other standard test guidelines, such as those of the ASTM or OECD). For example, most testing on fish was conducted for 24-hour durations instead of the typical 96-hour test duration reflecting the researcher's consideration of the rapid onset of mortality upon exposure to 6PPD. These deviations from standard testing methods made the derived toxicity values more uncertain and less in conformance with *Guidelines* methods.

However, in order to provide, states, authorized Tribes and other stakeholders with the best available information on the toxicity of 6PPD to aquatic organisms, the EPA developed an acute protective screening value for 6PPD in accordance with Section 304(a)(2) of the CWA. This work was undertaken to fulfill a pressing need to establish protective values for 6PPD

which has been found to be toxic to certain sensitive aquatic species, including sensitive fish. This screening value was calculated generally following the traditional (*Guidelines*) approach with limited adaptations, which are summarized in Section **Error! Reference source not found.**. GMAVs for the four most sensitive genera were within a factor of 5.7 of each other (Table 3-3). The freshwater Final Acute Value (FAV) (i.e., the 5th percentile of the genus sensitivity distribution, intended to address 95 percent of the genera) for 6PPD is 17.74 µg/L, calculated using the general approach described in the *Guidelines* (U.S.EPA 1985) (see Table 3-4, Figure 3-1). The FAV is lower than all the GMAVs for the tested species. The FAV was then divided by two to obtain a concentration yielding a minimal effects acute screening value (see Section **Error! Reference source not found.**). Based on the above, the FAV/2, which is the freshwater acute water column screening value, is 8.9 µg 6PPD/L (8,900 ng/L) (rounded to two significant figures). This 8,900 ng/L (parts per trillion) value is expected to be protective of 95% of freshwater genera exposed to 6PPD via direct aqueous (i.e., water-column) exposure, under short-term duration conditions of one-hour, when the screening value magnitude is not exceeded more than once in three years on average.

Table 3-3. Ranked Freshwater Genus Mean Acute Values.

Rank[a]	GMAV (µg/L 6PPD)	MDR Group[c]	Genus	Species	SMAV[b] (µg/L 6PPD)
1	28	C	*Oryzias*	Medaka, *Oryzias latipes*	28
2	94.94	B	*Gobiocypris*	Rare minnow, *Gobiocypris rarus*	94.94
3	143.7	A	*Oncorhynchus*	Coho salmon, *Oncorhynchus kisutch*	143.7
4	159.7	E	*Hyalella*	Amphipod, *Hyalella azteca*	159.7
5	213.4	D	*Daphnia*	Cladoceran, *Daphnia magna*	213.4
6	270.0	B	*Pimephales*	Fathead minnow, *Pimephales promelas*	270.0
7	299.0	G	*Lampsilis*	Wavy-eyed lampmussel, *Lampsilis fasciola*	156.0
				Fatmucket, *Lampsilis siliquoidea*	573.0
8	342.7	B	*Danio*	Zebrafish, *Danio rerio*	342.7

A Ranked from the most sensitive to the most resistant based on Genus Mean Acute Value (GMAV).
B From Appendix A: Quantitative Acute Freshwater Toxicity Data
c MDR Groups – Freshwater:

A. the family Salmonidae in the class Osteichthyes
B. a second family in the class Osteichthyes, preferably a commercially or recreationally important warmwater species (e.g., bluegill, channel catfish, etc.)
C. a third family in the phylum Chordata (may be in the class Osteichthyes or may be an amphibian, etc.)
D. a planktonic crustacean (e.g., cladoceran, copepod, etc.)
E. a benthic crustacean (e.g., ostracod, isopod, amphipod, crayfish, etc.)
F. an insect (e.g., mayfly, dragonfly, damselfly, stonefly, caddisfly, mosquito, midge, etc.)
G. a family in a phylum other than Arthropoda or Chordata (e.g., Rotifera, Annelida, Mollusca, etc.)
H. a family in any order of insect or any phylum not already represented.

Table 3-4. Freshwater Final Acute Value and Screening Value.

Calculated Freshwater FAV based on 4 lowest values: Total Number of GMAVs in Data Set = 8						
Rank	**Genus**	**GMAV (µg/L)**	**ln(GMAV)**	**ln(GMAV)^2**	**P=R/(N+1)**	**sqrt(P)**
1	*Oryzias*	28	3.332	11.104	0.1111	0.3333
2	*Gobiocypris*	94.94	4.533	20.73	0.2222	0.4714
3	*Oncorhynchus*	143.7	4.968	24.68	0.3333	0.5774
4	*Hyalella*	159.7	5.073	25.74	0.4444	0.6667
		Σ (Sum):	**17.93**	**82.25**	**1.111**	**2.049**

S^2 = 30.97 S = slope
L = 1.631 L = X-axis intercept
A = 2.876 A = lnFAV
FAV = 17.74 P = cumulative probability
SV = **8.9 µg/L (8,900 ng/L) 6PPD** (rounded to two significant figures)

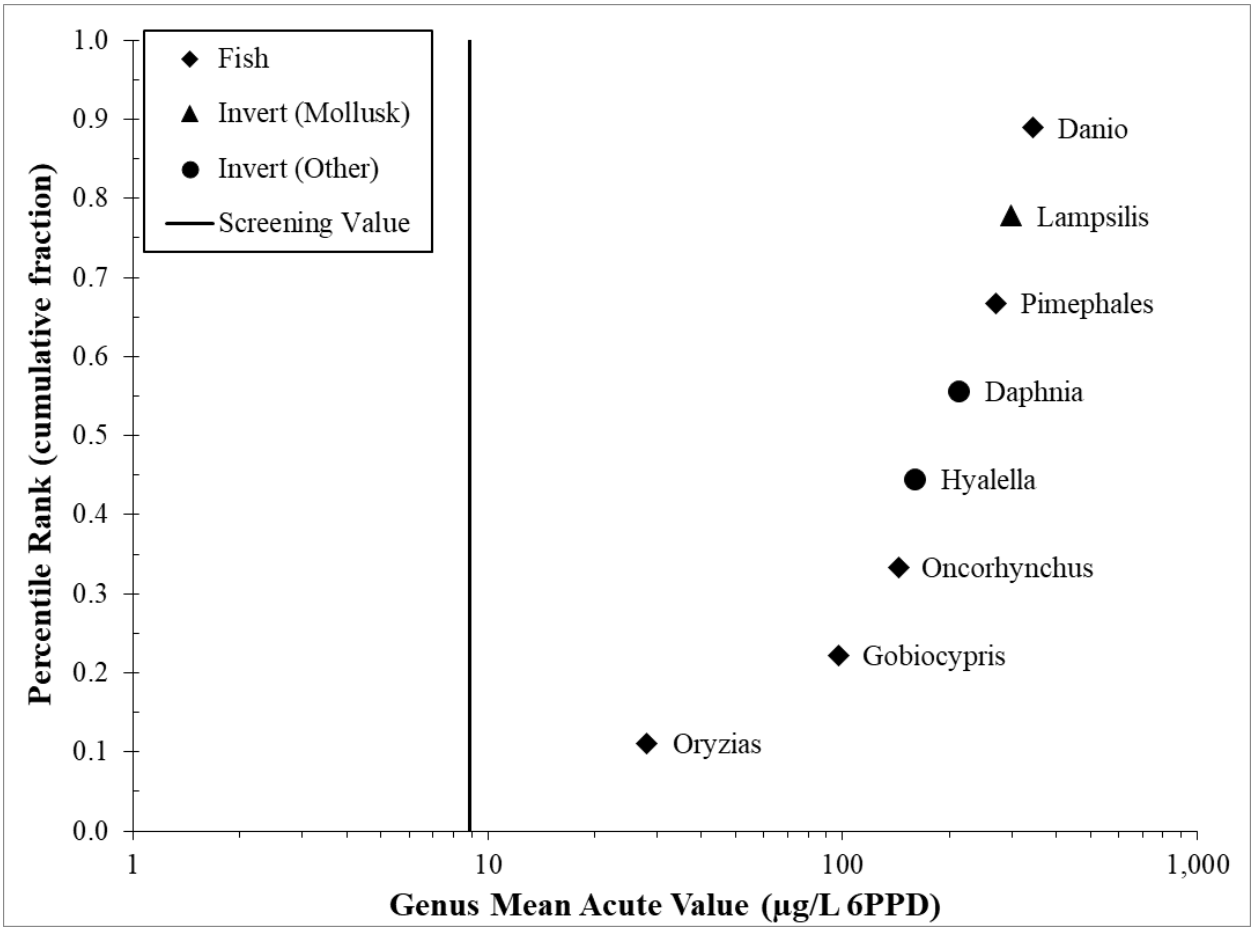

Figure 3-1. Ranked Freshwater 6PPD GMAVs Applicable to Fulfilling the Acute MDRs. The studies associated with the four most sensitive GMAVs are summarized above in Section 3.1.1 and studies associated with all quantitative GMAVs are summarized below in Appendix Section A.1.

3.2.2 Derivation of Acute Screening Value for Estuarine/Marine Water

No data exist to calculate an estuarine/marine FAV.

3.2.3 Derivation of Chronic Screening Value for Freshwater

Insufficient data exist to calculate a chronic freshwater FCV. However, the lowest chronic toxicity test result for medaka (*O. latipes*) was 6.38 µg/L, approximately equal to the acute screening value of 8.9 µg/L. Therefore,

3.2.4 Derivation of Chronic Screening Value for Estuarine/Marine Water

No data exist to calculate a chronic estuarine/marine FCV.

3.3 Summary of Acute 6PPD Freshwater Aquatic Life Screening Value

The aquatic life screening value for 6PPD derived in this document includes a water-column based acute screening value for freshwaters. A chronic screening value for freshwaters and acute and chronic water column screening values for estuarine/marine waters could not be derived at this time due to data limitations. However, given the short half-life of 6PPD and the rapid mortality of test organisms in studies across several species, acute toxicity is expected to be a more important driver for aquatic risk than chronic toxicity. The screening value for acute exposures of 6PPD in freshwater is 8.9 µg/L (8,900 ng/L) (Table 3-5). As part of deriving the screening value for 6PPD, the EPA made several adaptations to the traditional (*Guidelines*) approach. These adaptations related to the use of atypical acute study designs and the relatively limited data previously noted inherently make the screening value less certain than criteria derived using the traditional (*Guidelines*) approach. The screening value for 6PPD provides information that states and Tribes can consider in their water quality protection programs. The screening value concentrations are expected to be generally protective of 95% of freshwater species potentially exposed to 6PPD for short durations (e.g., one hour or less). The science and

understanding of the aquatic toxicity of 6PPD is relevantly recent (with 6PPD transformation product 6PPD-q being attributed as the causative pollutant behind urban runoff mortality syndrome (URMS) in the past decade), and a number of toxicity studies are currently underway. As such, the EPA will continue to monitor the 6PPD literature and toxicity data to evaluate the protectiveness of this screening value. This screening value is expected to be protective if not exceeded for more than one hour every three years, using the standard acute criteria duration and frequency parameters.

Table 3-5. Freshwater 6PPD Aquatic Life Screening Value.

Type/Media	Acute Water Column Screening Value[1]
Magnitude	8.9 µg/L (8,900 ng/L)
Duration	One hour average
Frequency	Not to be exceeded more than once in three years on average

[1] Applicable throughout the water column.

4 EFFECTS CHARACTERIZATION

4.1 Additional Analyses Supporting the Derivation of the Screening Value for Acute Exposures of 6PPD in Freshwater

In addition to the EPA's screening value for acute exposures of 6PPD in freshwater of 8.9 µg/L described above in Section 3.3, an additional analysis was completed as part of an evaluation to examine the effect of using additional qualitative data in the calculation of the *Oncorhynchus* GMAV on the magnitude of the 6PPD screening value (Table 4-1).

The additional analysis presented below recalculated the *Oncorhynchus* GMAV as the geometric mean of the quantitative *Oncorhynchus kisutch* test conducted by Tian et al. (2021) described above and a qualitative test with a non-definitive value for *Oncorhynchus mykiss* conducted by Nair et al. (2023). This qualitative value was excluded from the quantitative analysis because the test represents an unbounded (greater than) low effect value when compared to the acceptable quantitative acute value for *Oncorhynchus* species, consistent with previous practice (U.S. EPA 2013). The additional analysis presented here is solely intended to support the screening value for acute exposures to 6PPD in freshwater through a weight-of-evidence approach that evaluated the influence of data variation on the screening value derivation process.

Table 4-1. Additional Analyses Supporting the Derivation of 6PPD Screening Value for Comparative Purposes.

Purpose of Additional Analysis	Details of Additional Analysis	Calculated Acute Water Column Concentration for Additional Analysis (µg 6PPD/L)	*Oncorhynchus* References Used in both Analyses
To examine the effect of including a qualitative *Oncorhynchus mykiss* value on the magnitude of the 6PPD screening value	An *Oncorhynchus* GMAV of 99.58 µg/L 6PPD was calculated from the two SMAVs (of 143.7 and >69 µg/L 6PPD)	9.0	Nair et al. (2023); Tian et al. (2021)
Screening Value for Acute Exposures of 6PPD in Freshwater		**8.9 µg 6PPD/L**	

For this additional analysis, an *Oncorhynchus* GMAV of 99.58 µg 6PPD/L was used instead of a GMAV of 143.7 µg/L 6PPD from a single study (Tian et al. 2021) as was done for the screening value. This additional GMAV is based on two *Oncorhynchus* species: *O. kisutch* and *O. mykiss*. The acute water column value calculated via this additional analysis yielded a value of 9.0 µg/L, which is nearly identical to the EPA's recommended acute screening value. Based on this additional analysis, the EPA decided to retain the qualitative classification of the unbounded *O. mykiss* test.

4.2 Qualitative Study Summaries

Several studies were identified as not meeting the EPA's data quality considerations for inclusion in the quantitative dataset for the acute screening value. These studies were used qualitatively as supporting information to provide additional evidence of the protection afforded freshwater aquatic organisms from adverse short-term effects of 6PPD. The key studies with apical endpoints (e.g., acute mortality) used qualitatively in support of the acute 6PPD screening value are summarized below, sorted by taxonomic relatedness to the corresponding genera with available quantitative data (e.g., sensitive fish, such as Japanese medaka, listed first followed by aquatic invertebrates and all ranked according to sensitivity). NOEC and LOEC values were provided in several of the following study summaries as representative toxicity values for comparison to the quantitative acute toxicity values summarized in the Effects Analysis section above (see Section **Error! Reference source not found.**). None of the following values were used quantitatively due to shortcomings in the studies, such as tests that were conducted with a single concentration of 6PPD resulting in no observed effects.

Figure 4-1 presents both the quantitative (filled symbols) data summarized in Section 3.1.1 and qualitative (open symbols) data from studies that are summarized in Table 4-2 and

Section **Error! Reference source not found.**. The presentation of both the quantitative and qualitative 6PPD data are intended to demonstrate two key points regarding the protectiveness of the screening value presented in this document. First, Figure 4-1 illustrates that fish appear to be the most sensitive genera to acute exposures of 6PPD. However, unlike 6PPD-q, salmonids do not appear to be particularly sensitive. Second, the presentation of both the quantitative and qualitative data displays the relative sensitivities of other aquatic organisms and the inherent protectiveness of this screening value on aquatic life in freshwater, although the qualitative data are more uncertain.

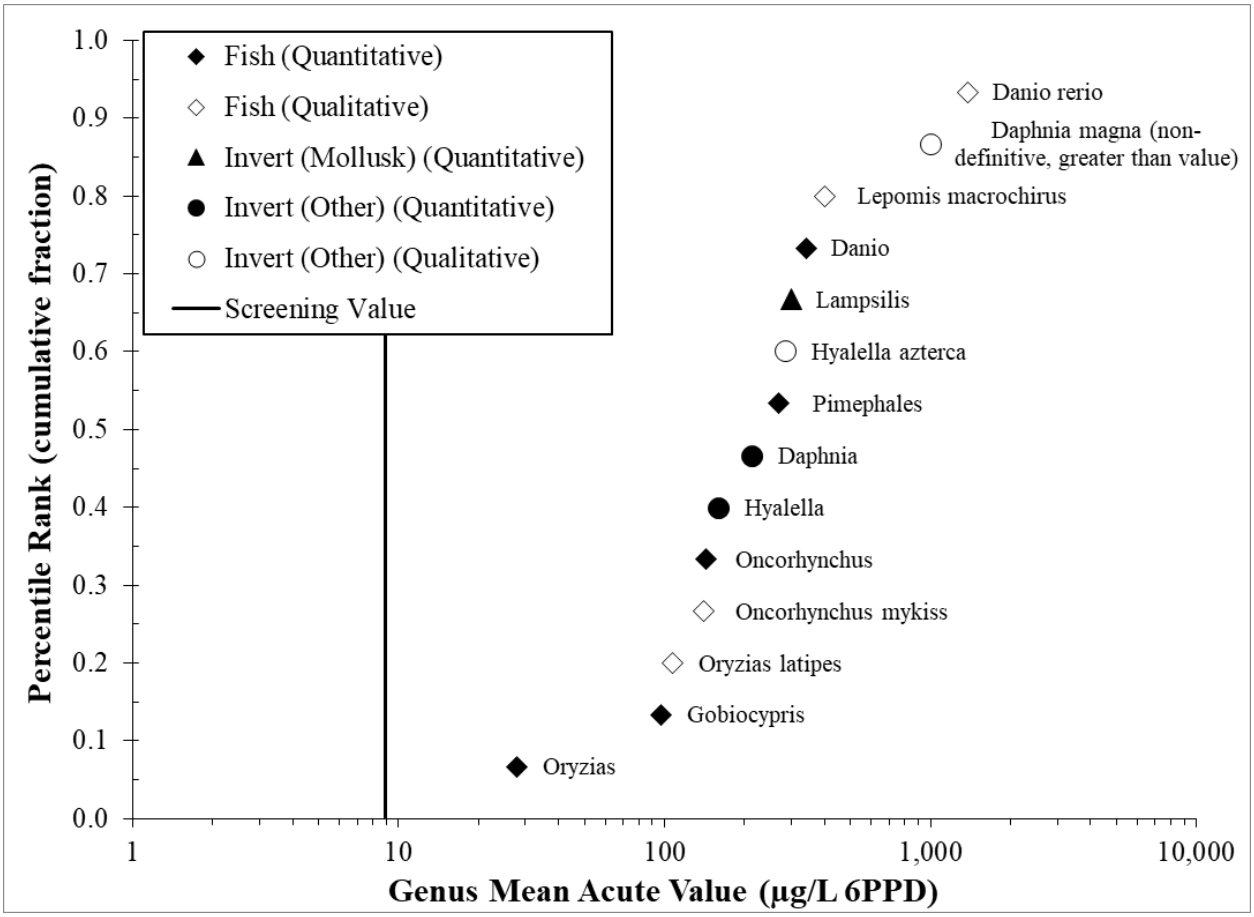

Figure 4-1. Screening Value and Aquatic Life Sensitivity Distribution for 6PPD in Freshwater.

The quantitative studies were summarized above in Section 3.1.1 and Appendix A, and the qualitative studies are summarized below. Filled symbols reflect ranked quantitative mean acute values while open symbols reflect qualitative values.

Table 4-2. Qualitative Data Used as Supporting Information in the Derivation of the Acute Value for 6PPD in Freshwater.
Ensuing study summaries are below. Underlined text highlights critical deficiencies of the study.

Taxonomic Group	Phylum	Family	Species (lifestage)	Method[a]	Test Duration	Chemical/ Purity	Biomass Loading Rate (g/L)[b]	Effect	Reported Effect Concentration (µg/L)	Deficiencies	Reference
Qualitative Data for Genera with Quantitative Data											
Fish	Chordata	Adrianichthyidae	Medaka (41 d), *Oryzias latipes*	R, M	96 hr	6PPD >98%		80% mortality	<107	Only one exposure concentration resulting in no definitive effect value, greater than high value[e]; no replication[c] with 10 organism per treatment	Hiki et al. (2021)
Benthic crustacean	Arthropoda	Hyalellidae	Amphipod (3-5 d), *Hyalella azteca*	R, M	96 hr	6PPD >98%	N/A	100% mortality	286	Only one exposure concentration resulting in no definitive effect value, a less than high value[e];	Hiki et al. (2021)
Planktonic crustacean	Arthropoda	Daphniidae	Cladoceran (<24 hr), *Daphnia magna*	S, U	48 hr	N-(1,3-dimethylbutyl)-N'-phenyl-1,4-benzenediamine Not reported	N/A	EC_{50} (immobility)	>1,000 (aged for 24 hours)	Only three exposure treatments	Monsanto Co. (1984)
Fish	Chordata	Cyprinidae	Zebrafish (embryo, <3 hpf), *Danio rerio*	R, M	96 hr	6PPD >98%		LC_{50}	> 137	Only one exposure concentration resulting in no definitive effect value, a greater than low value[e];	Hiki et al. (2021)
Fish	Chordata	Cyprinidae	Zebrafish (adult, 4 mo), *Danio rerio*	S, M	12 hr	6PPD 98.0%		LOEC (swimming speed and distance)	1,000	Duration too short (12 hours)[d]; non-apical endpoint	Ji et al. (2022)
Fish	Chordata	Cyprinidae	Zebrafish (embryo, 2 hpf), *Danio rerio*	R, U	96 hr	6PPD >99.0%		LC_{50}	2,200	Limited test details; number of organisms per replicate uncertain. Use of controls assumed.	Peng et al. (2022)

Taxonomic Group	Phylum	Family	Species (lifestage)	Method[a]	Test Duration	Chemical/ Purity	Biomass Loading Rate (g/L)[b]	Effect	Reported Effect Concentration (µg/L)	Deficiencies	Reference
Fish	Chordata	Cyprinidae	Zebrafish (embryo, 8 hpf), *Danio rerio*	R, U	112 hr	6PPD >98%		NOEC (mortality)	1,200	Atypical test duration	Zhang et al. (2023)
Qualitative Data for Fish Genera without Quantitative Data											
Fish	Chordata	Salmonidae	Rainbow trout *Oncorhynchus mykiss*	S, U	96 hr	N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine Not reported		LC_{50}	140	Only ten fish tested with no replication[c]. Missing exposure details (dilution water in particular); Results of one test reported but two different test procedures included	Monsanto Co. (1977)
Fish	Chordata	Salmonidae	Rainbow trout (juvenile, 2 mo), *Oncorhynchus mykiss*	S, M	96 hr	6PPD Not reported		LC_{50}	> 69.0	Not definitive effect value, a greater than low value[e];	Nair et al. (2023)
Fish	Chordata	Centrarchidae	Bluegill, *Lepomis macrochirus*	S, U	96 hr	N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine Not reported		LC_{50}	400	Only ten fish tested with no replication[c]. Missing exposure details (dilution water in particular); Results of one test reported but two different test procedures included	Monsanto Co. (1977)

[a] S=static, R=renewal, F=flow-through, U=unmeasured, M=measured

[b] The EPA's 2016 Ecological Effects Test Guidelines (OCSPP 850.1075) for Freshwater and Saltwater Fish Acute Toxicity Tests recommend that biomass loading should be ≤ 0.8 g wet weight (ww) per liter (g/L) in static or static-renewal tests and ≤ 0.5 g/L per 24 hours and < 5 g/L at any time in flow-through tests.

[c] The EPA's 2016 Ecological Effects Test Guidelines (OCSPP 850.1075) for Freshwater and Saltwater Fish Acute Toxicity Tests state that there be a minimum of 7 test organisms and 1 replicate test vessel per exposure treatment, with 10 test organisms and 2 replicate test vessels preferred.

[d] The EPA's 2016 Ecological Effects Test Guidelines (OCSPP 850.1075) for Freshwater and Saltwater Fish Acute Toxicity Tests state that the test duration should be 96 hours.

[e] Consistent with past practice, a decision rule was applied to the 6PPD toxicity data as follows: "greater than" (>) high toxicity values and "less than" (<) low toxicity values were included (U.S.EPA 2013).

4.2.1 Consideration of Qualitatively Acceptable Acute Data

4.2.1.1 Qualitatively Acceptable Acute Data for Species Among the Four Most Sensitive Genera with Quantitative Data

4.2.1.1.1 Genus Oryzias

Hiki et al. (2021) performed a 96-hour static-renewal, measured test of 6PPD (>98% purity) with the Japanese medaka, *Oryzias latipes*. The acute toxicity test followed OECD guideline number 203 (OECD 2019). No mortality was observed in the control and 80% mortality was observed in the single treatment concentration, resulting in a 96-hour LC_{50} of <107 µg/L. The value was considered acceptable for qualitative use because there was only one test concentration, and the resulting concentration was not definitive.

4.2.1.1.2 Genus Oncorhynchus

Monsanto Co. (1977) performed a 96-hour static, unmeasured acute test of 6PPD (purity not reported; assumed to be high purity [> 98%) since the test was part of OECD's 6PPD assessment (OECD 2004a)] on rainbow trout, *Oncorhynchus mykiss*. Acute tests were conducted following an in-house protocol. Mortality was observed after 24 hours in the three highest test concentrations, ranging from 30-100%. At 96 hours mortality was ≥10% across all 6PPD test concentrations except 0.087 mg/L, where no mortality was observed. No mortality was observed in negative or solvent controls. The 96-hour LC_{50} was calculated as 140 µg/L 6PPD. Since the dilution water was not reported and dissolved oxygen decreased to unacceptable levels in the test, the result was considered qualitative.

Nair et al. (2023) conducted a 96-hour measured, static acute test of 6PPD (purity not provided, purchased from the Toronto Research Chemicals, Toronto, ON, Canada) with rainbow trout, *Oncorhynchus mykiss*. The author-reported 96-hour LC_{50} was >69.0 µg 6PPD/L and was excluded from quantitative studies because it is a greater than low value compared to other *Oncorhynchus* effect concentrations.

4.2.1.1.3 Genus Hyalella

Hiki et al. (2021) performed a 96-hour static-renewal, measured test of 6PPD (>98% purity) with the amphipod, *Hyalella azteca*. The acute toxicity test followed the test method outlined by Environment and Climate Change Canada (2017)(ECCC 2017). Mortality in the control was 5%, and mortality in the single test concentration was 100%. The 96-hour LC_{50} was <286 µg/L. The value was considered acceptable for qualitative use because there was only one test concentration, and the effect concentration was not definitive.

4.2.1.2 Qualitatively Acceptable Acute Data for Other Genera Used as Supporting Information

4.2.1.2.1 Genus Daphnia

Monsanto Co. (1984) performed a 48-hour static, unmeasured acute test of 6PPD [purity not reported; assumed to be high purity (> 98%) since the test was part of OECD's 6PPD assessment (OECD 2004a)] on the cladoceran, *Daphnia magna*. Acute tests were conducted immediately after introducing the chemical to well water and after the 6PPD in solution had been aged for 24 hours. The quantitatively acceptable unaged study is described below (Appendix Section A.2.5), and the qualitatively acceptable aged study is described here. Toxicity tests followed MIC Environmental Services, *Environmental Assessment Method for Conducting Acute Toxicity Tests with Daphnia magna* (Grueber and Adams 1980) and *Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians* (U.S.EPA 1975). Zero percent mortality was observed across all treatments (i.e., no effect up the nominal concentration of 1,000 µg/L 6PPD), but since the study was unmeasured and the half-life of 6PPD was less than 24 hours the actual test concentrations are unknown, and therefore, the test result for this study was considered acceptable for qualitative use only at this time.

4.2.1.2.2 Genus Danio

Hiki et al. (2021) performed a 96-hour static-renewal, measured test of 6PPD (>98% purity) with the zebrafish, *Danio rerio*. The acute toxicity test followed OECD guideline number 236 (OECD 2013). Embryos (16 cell stage) used for testing were obtained from brood stock maintained at the National Institute of Environmental Studies. The test was conducted in 24-well plates, with 2 mL test solution added per plate. One embryo was added to each well, for a total of 20 embryos each for the negative control and treatment, respectively. No mortality was observed in the control or single treatment concentration resulting in a 96-hour LC_{50} is >137 µg/L. This value was considered acceptable for qualitative use because there was only one test concentration, and the value was not definitive.

Ji et al. (2022) performed a 12-hour static, measured test of 6PPD (98.0% purity) with zebrafish, *Danio rerio*. Swimming velocity decreased by 42.4% at 1,000 µg/L when compared to the control, which was statistically significant, resulting in a 12-hour LOEC of 1,000 µg/L 6PPD. The test was considered qualitative because it was of insufficient duration for an acute toxicity test and was based on a non-apical behavioral endpoint.

Peng et al. (2022) performed a 96-hour static-renewal, unmeasured test of 6PPD (>99.0% purity) with zebrafish, *Danio rerio*. The test was carried out according to Organization for Economic Co-operation and Development (OECD) test guideline 236 (fish embryo toxicity test). During the experimental period, the exposure solutions were renewed every 24 hours. The 96-hour LC_{50} was calculated as 2,200 µg/L 6PPD. The test was considered qualitative because of insufficient information regarding organism number, replication, and uncertainty regarding the use of controls and control response.

Zhang et al. (2023) conducted a 112-hour static-renewal, unmeasured acute test 6PPD (>98% purity) with zebrafish, *Danio rerio*. Zebrafish embryos at 8 hpf were continuously

exposed to 6PPD during the embryo development stage till 120 hpf, with solutions changed at 60 hpf. Twelve-well plates were used to carry 20 embryos and 3 mL exposure solution per well. There were 20 embryos per replicate and three biological triplicates per group. The exposure plate was covered with foil to avoid photolysis. The embryo hatching rate at 48 and 72 hpf and accumulated malformation and mortality at 120 hpf were recorded. Between 24 and 120 hpf, embryo development was recorded daily under the dissecting microscope, and malformations such as pericardial edema, yolk sac edema, uninflated swim bladder, eye and pigment abnormity were imaged and counted. At 120 hpf, the accumulated malformation and mortality was calculated using triplicates. The author-reported 112-hour mortality NOEC was 1,200 µg 6PPD/L, which was acceptable for qualitative use due to atypical test duration.

4.2.1.2.3 Genus Lepomis

Monsanto Co. (1977) performed a 96-hour static, unmeasured acute test of Santoflex 13 [N-(1,3-dimethylbutyl)-N'-phenyl-1,4-benzenediamine (6PPD, CAS # 793-24-8, purity not reported] on bluegill sunfish, *Lepomis macrochirus*. Mortality was observed after 24 hours in the two highest test concentrations, ranging from 30-100%. At 96 hours mortality was ≥10% across all 6PPD test concentrations except 0.24 mg/L, which observed no mortality. No mortality was observed in negative or solvent controls. The 96-hour LC_{50} was reported as 400 µg/L 6PPD. Since the dilution water was not reported and dissolved oxygen decreased to unacceptable levels in the test, the result was considered qualitative.

4.2.2 Summary of Qualitatively Acceptable Acute 6PPD Toxicity Studies for Estuarine/Marine Species

There were no qualitatively acceptable empirical data for acute 6PPD toxicity for estuarine/marine species at the time of the literature review (completed in December 2023).

4.2.3 Summary of Qualitatively Acceptable Chronic 6PPD Toxicity Studies for Freshwater Species

The four toxicity values summarized in Appendix F were not used either quantitatively or qualitatively to derive the chronic 6PPD screening value for aquatic life in freshwater due to shortcoming in the studies, such as the use of sediments and the testing of a single concentration of 6PPD resulting in no observed effects. Results of each individual study and the rationale for why the studies were not used are presented in Appendix F.2.1.

4.2.4 Summary of Qualitatively Acceptable Chronic 6PPD Toxicity Studies for Estuarine/Marine Species

There were no qualitatively acceptable empirical data for chronic 6PPD toxicity for estuarine/marine species at the time of this literature review (completed in December 2023).

4.3 Protection of Threatened and Endangered Species

Although the 6PPD acute and chronic datasets are not extensive, the acute dataset does include some data representing species that are listed as threatened or endangered by the U.S. Fish and Wildlife Service and/or National Oceanic and Atmospheric Administration (NOAA) Fisheries. Brief summaries are provided in the sub-sections below describing the available 6PPD toxicity data for listed species indicating that the 2024 freshwater acute 6PPD screening value is expected to be protective of these species, based on currently available scientific data.

4.3.1 Quantitative Acute Toxicity Data for Listed Species

Quantitative freshwater acute toxicity test data from studies evaluating the effects of 6PPD on threatened and endangered freshwater species were available for coho salmon (*Oncorhynchus kisutch*) with a SMAV of 143.7 µg/L 6PPD (Tian et al. 2021). The coho salmon SMAV is 16 times higher than the acute screening value of 8.9 µg/L. There were no quantitative freshwater chronic toxicity data, or quantitative estuarine/marine acute or chronic data, for

endangered or threatened aquatic species at the time of the literature review (completed in December 2023).

4.3.2 Qualitative Acute Toxicity Data for Listed Species

Qualitative acute freshwater toxicity data from studies evaluating the effects of 6PPD were available for rainbow trout (*Oncorhynchus mykiss*). Monsanto Co. (1977) reported a 96-hour LC_{50} of 140 µg/L, and Nair et al. (2023) reported a 96-hour LC_{50} of >69.0 µg/L, both of which are substantially greater than the screening value of 8.9 µg/L. There were no qualitative freshwater chronic toxicity data, or qualitative estuarine/marine acute or chronic data, for endangered or threatened aquatic species at the time of the literature review (completed in December 2023).

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Appendix A Quantitative Acute Freshwater Toxicity Data

A.1 Summary Table of Acceptable Quantitative Acute Freshwater 6PPD Toxicity Data

Values used for SMAV calculations are highlighted in bold.

Species (lifestage)	Method[a]	Test Duration	Chemical / Purity	pH	Temp. (°C)	Hardness (mg/L as $CaCO_3$)	Effect	Author Reported Effect Conc. (µg/L)	EPA Adjusted / Calculated LC_{50} (µg/L)	Species Mean Acute Value (µg/L)	Reference
Wavy-rayed lampmussel (glochidia), *Lampsilis fasciola*	S, M	24 hr	6PPD >95%	8.1-8.2	20	126	EC_{50} (viability)	260	**156.0**	156.0	Prosser et al. (2017b)
Fatmucket (glochidia), *Lampsilis siliquoidea*	S, M	24 hr	6PPD >95%	7.78-8.44	20	126	EC_{50} (viability)	955	**573.0**	573.0	Prosser et al. (2017b)
Cladoceran (<24 hr), *Daphnia magna*	S, U	48 hr	6PPD Not reported	7.6-8.3	22	218-274	EC_{50} (immobility)	510	**306.0**	-	Monsanto Co. (1984)
Cladoceran, *Daphnia magna*	S, M	48 hr	6PPD Not reported	-	-	-	EC_{50}	**230**	-	-	Japan Ministry of the Environment (2019)
Cladoceran (<24 hr), *Daphnia magna*	S, M	48 hr	6PPD >98%	8.00-8.41	21.9	-	EC_{50} (immobility)	**<138**	-	213.4	Hiki et al. (2021)
Amphipod (juvenile, 7-11 d), *Hyalella azteca*	S, M	96 hr	6PPD >98.0%	8.18-8.38	23	126	LC_{50}	250	**159.7**	159.7	Prosser et al. (2017a)
Coho salmon (juvenile, 0-2 yr), *Oncorhynchus kisutch*	S, U	24 hr	6PPD 95%	7.6-7.8	10.0-12	-	LC_{50}	251	**143.7**	143.7	Tian et al. (2021)
Zebrafish (embryo), *Danio rerio*	R, U	96 hr	6PPD >98%	6.81	26	-	LC_{50}	442.62	**265.6**	-	Varshney et al. (2022)
Zebrafish (embryo, 2 hpf), *Danio rerio*	R, U	94 hr	6PPD >98%	7.0-7.3	28	100-200	LC_{50}	737	**442.2**	342.7	Fang et al. (2023)
Rare minnow, *Gobiocypris rarus*	R, M	96 hr	6PPD ≥98%	-	25	-	LC_{50}	162	**94.94**	94.94	Di et al. (2022)

Species (lifestage)	Method[a]	Test Duration	Chemical / Purity	pH	Temp. (°C)	Hardness (mg/L as $CaCO_3$)	Effect	Author Reported Effect Conc. (µg/L)	EPA Adjusted / Calculated LC_{50} (µg/L)	Species Mean Acute Value (µg/L)	Reference
Fathead minnow, *Pimephales promelas*	F, M	96 hr	6PPD Not reported	7.7-7.9	21-22	250	LC_{50}	450	**270.0**	270.0	Monsanto Co. (1979)
Medaka, *Oryzias latipes*	S, M	96 hr	6PPD Not reported	-	-	-	LC_{50}	**28**	-	28	Japan Ministry of the Environment (2019)

a S=Static, R=static-renewal, F=Flow-through, U=unmeasured, M=measured

A.2 Detailed 6PPD Acute Freshwater Toxicity Study Summaries and Corresponding Concentration-Response Curves (when calculated for the most sensitive genera)

This appendix presents detailed study summaries for tests that were considered quantitatively acceptable for screening value derivation. Study summaries are presented below in order of taxonomic sensitivity to 6PPD. Concentration-response (C-R) models developed by the EPA that were used to determine acute toxicity values used for screening value derivation are also presented for the most sensitive genera/species when available. C-R models included here with study summaries were those for the four most sensitive genera. In many cases, authors did not report C-R data in the publication/supplemental materials and/or did not provide C-R data upon the EPA's request. In such cases, the EPA did not independently calculate a toxicity value and the author-reported effect concentrations were used in the derivation of the screening value.

A.2.1 Most acutely sensitive genus: *Oryzias* (Medaka)

Ministry of the Environment, Japan (2019) performed a 96-hour static, measured acute test (based on study details provided in OECD (2004a)) of N-(1,3-dimethylbutyl)-N'-phenyl-1,4-benzenediamine (6PPD, CAS # 793-24-8, purity not reported) with the Japanese medaka, *Oryzias latipes*. This species is not a North American resident species, but it is a common aquatic toxicity test species that serves as a surrogate for untested fish species residing in North America. The toxicity test method used followed OECD TG 203 (Fish Acute Toxicity Test) (OECD 1992). No details were provided with regards to the source of the fish, preparation of test solutions, and exposure conditions. Adequate control survival and other test acceptability requirements were assumed per OECD test guidelines. The author-reported 96-hour LC_{50} for the test was 28 µg/L. It was unclear when the test concentration measurements were taken (assumed to be average concentrations). Therefore, the author-reported value was used. The value was considered acceptable for quantitative use despite some missing exposure and test details, given that the test

was conducted by the Ministry of the Environment in Japan and followed OECD test guidelines. These details included the lack of information on the chemical purity of 6PPD used in the toxicity test. The 6PPD used was assumed to be high purity (> 98%) since the test followed OECD test guidelines and was used in OECD's 6PPD assessment (OECD 2004a). Further, it appeared the source of the 6PPD was Bayer AG and OECD (2004a) states: "*In Germany 6PPD is manufactured in an industrial scale only at the Bayer AG Brunsbüttel plant. In a continuously working closed system 4-aminodiphenylamine is reacted with an excess of methyl isobutyl ketone (MIBK) to a Schiff´s base. This base is then hydrogenated catalytically. The excess of MIBK is separated off. The hydrogenation by-products are purged with steam. Impurities are removed by distillation under reduced pressure yielding 6PPD with a purity of > 98 %.*"

A.2.1.1 Japan Ministry of the Environment (2019) Concentration-Response Curve – Oryzias latipes (Medaka)

Publication: Japan Ministry of the Environment 2019
Species: *Oryzias latipes*
EPA-Calculated LC_{50}: Not calculable, C-R data not available

A.2.2 Second most acutely sensitive genus: *Gobiocypris* (rare minnow)

Di et al. (2022) performed a 96-hour static-renewal, measured acute test of N-(1,3-dimethylbutyl)-N′ -phenyl-p-phenylenediamine (6PPD, ≥ 98% purity, obtained from J&K Scientific Ltd.) with the rare minnow, *Gobiocypris rarus*. This species is not a North American resident species, but it is a common aquatic toxicity test species that serves as a surrogate for untested fish species residing in North America. The acute test followed OECD 203 methodology (OECD 1992). Fish (0.18 g) used for testing were obtained from CASA Zhongke Water Quality Co., Ltd. and were acclimated for two weeks before testing. Eight fish were added to each test vessel containing 1.5 L of solution, and test solutions were renewed every 12 hours over the course of the experiment. Each test treatment was replicated three times. Solvent

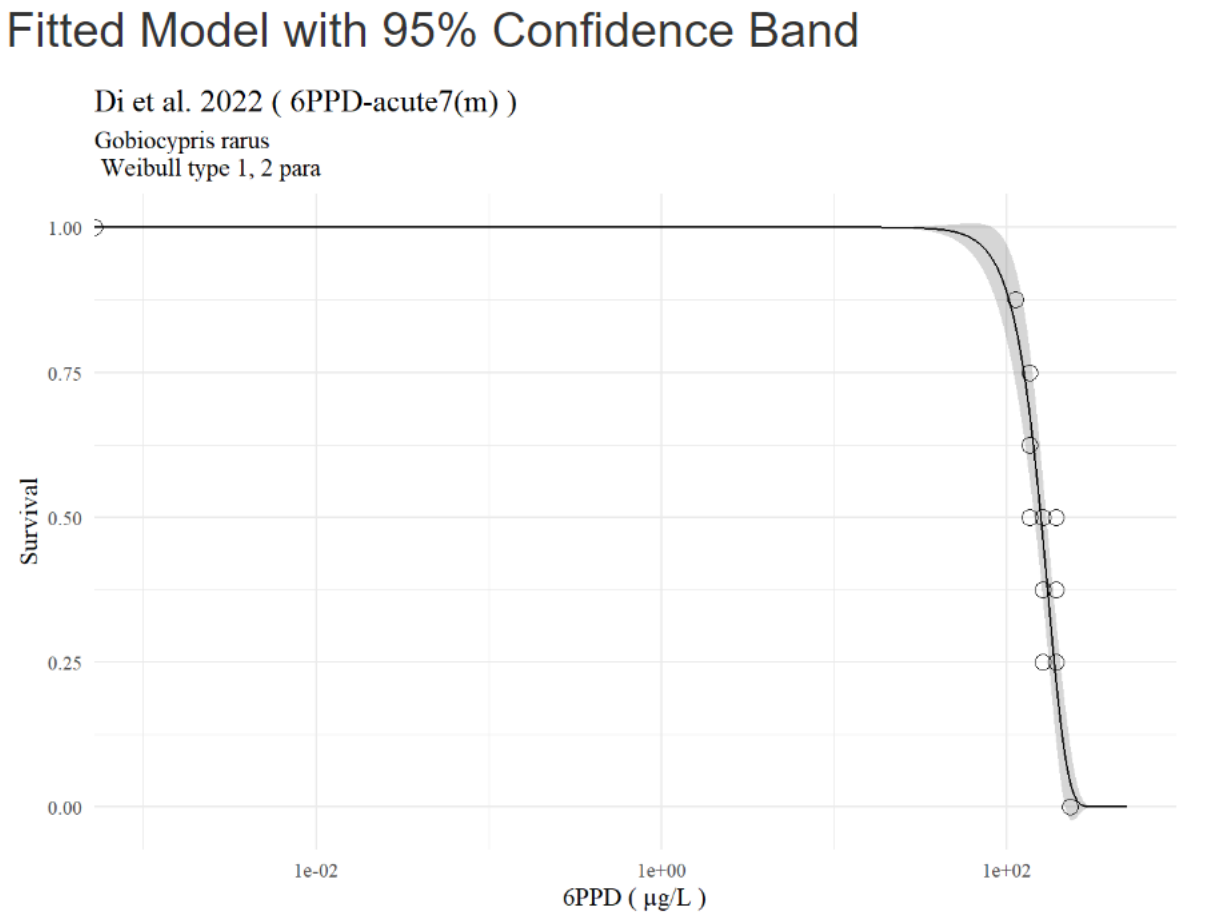
controls were maintained in dechlorinated tap water and acetonitrile (solvent volume not provided) and there were five test treatments (112, 135, 162, 194 and 233 µg/L measured 6PPD). Tests were conducted at 25±1°C and a 14 hour light photoperiod. The author-reported 96 hour LC_{50} was 162 µg/L 6PPD. The EPA calculated an LC_{50} (158.23 µg/L) based on measured concentrations provided by the study authors; however, it was unclear when these measurements were taken during the exposure. It was assumed the measured concentrations provided were initial concentrations. The EPA-calculated LC_{50} value was adjusted to lower the value by 40% in order to account for loss of 6PPD over experiment duration. The adjusted EPA-calculated LC_{50} was 94.94 µg/L 6PPD, which was acceptable for quantitative use.

A.2.2.1 Di et al. (2022) Concentration-Response Curve – Gobiocypris rarus (Rare minnow)

Publication: Di et al. 2022
Species: *Gobiocypris rarus*
EPA-Calculated LC_{50}: 158.23 µg/L (or 94.94 µg/L after adjusting for loss)
Concentration-Response Model Estimates:

Parameter	Estimate	Std. Error	t-stat	p-value
b	3.8787	0.6731	5.762	$8.306e^{-9}$
e	173.91	6.3459	27.41	$2.2e^{-16}$

Concentration-Response Model Fit:



A.2.3 Third most acutely sensitive genus: *Oncorhynchus* (salmon)

Oncorhynchus kisutch

Tian et al. (2021) performed a 24-hour static, unmeasured test of N-(1,3-dimethylbutyl)-N′ -phenyl-p-phenylenediamine (6PPD, CAS# 793-24-8, 95.0% purity, obtained from Usolf Chemicals (Shandong, China)) with juvenile (0-2 yr) coho salmon, *Oncorhynchus kisutch*. Coho salmon from Soos Creek or Diru Creek stock reared at the Puyallup Research and Extension Center of Washington State University were used for testing. Dilution water was dechlorinated municipal water treated by reverse osmosis and reconstituted with buffered Instant Ocean salts to approximately pH 7.5 and 1,300 µS/cm conductivity at 10-13°C. Individual coho salmon used in experiments were age 0+ or 1+ yr (1.3-28.0 g). A controlled-dose fish exposure to 6PPD was

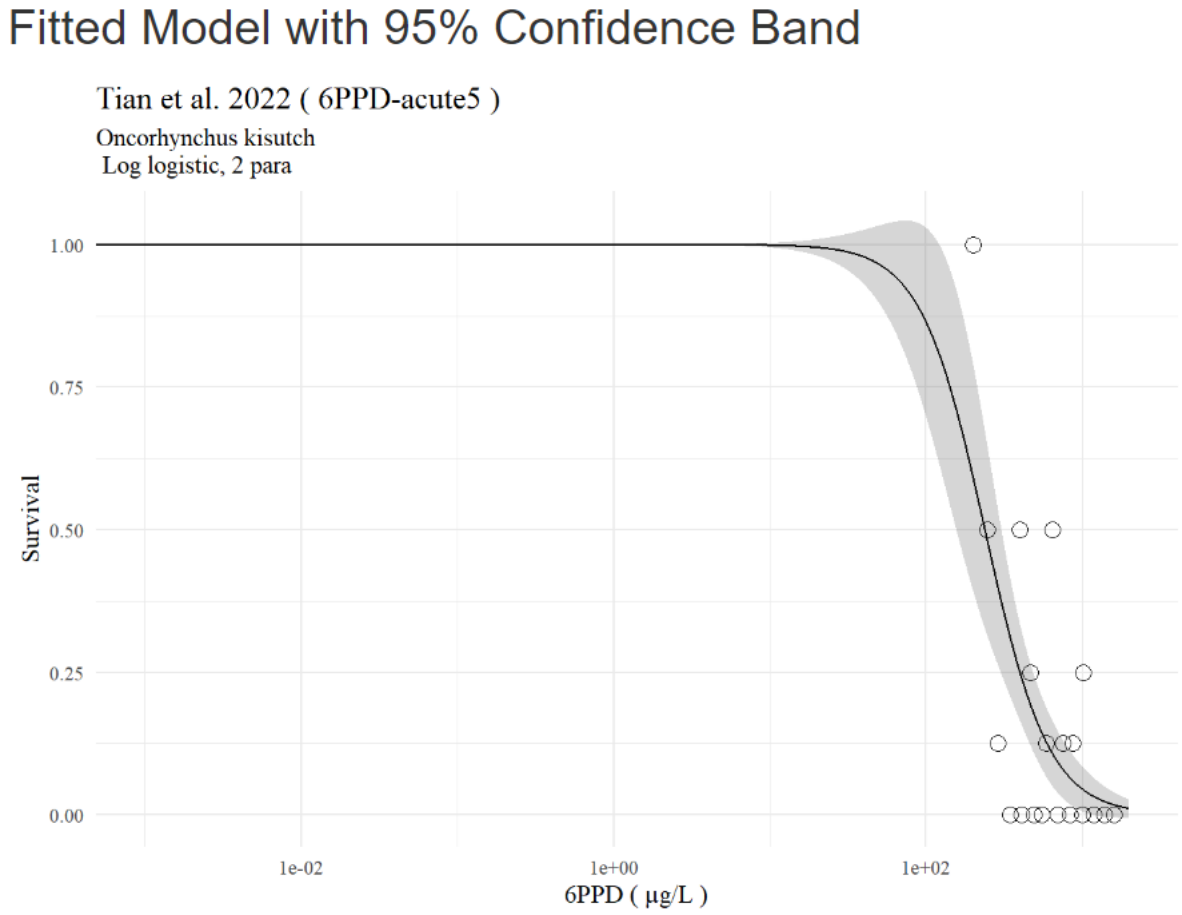
used as a screening exposure for the study. The screening exposure involved exposing eight fish per concentration in 30 L of test solution, with a 10x-concentration dilution series. A solvent control (material and amount by volume not provided) and a positive control (250 mg/L tire wear particle leachate) were additionally included. The exposure was repeated twice. While there was no information in the publication about time of death or immobility in the definitive 6PPD exposure, authors noted that exposures to ozone-synthesized and tire leachate-derived 6PPD-q (~20 mg/L nominal concentrations) induced rapid mortality within five hours and initial symptoms (locomotor deficiencies) were evident within 90 minutes. The author-reported 24-hour LC_{50} was calculated as 251 µg/L based on nominal concentrations of 6PPD. The EPA calculated an LC_{50} of 239.56 µg/L based on the C-R data reported in the publication. Since test concentrations were unmeasured, the EPA-calculated LC_{50} value was adjusted to lower the value by 40% in order to account for loss of 6PPD over experiment duration. The adjusted EPA-calculated LC_{50} was 143.7 µg/L 6PPD and was acceptable for quantitative use despite the short duration (24 hours opposed to 96 hours), as this duration was considered acceptable since it represented realistic exposures in the aquatic environment.

A.2.3.1 Tian et al. (2021) Concentration-Response Curve – Oncorhynchus kisutch (Coho salmon)

Publication: Tian et al. 2021
Species: *Oncorhynchus kisutch*
EPA-Calculated LC_{50}: 239.56 µg/L (or 143.7 µg/L after adjusting for loss)
Concentration-Response Model Estimates:

Parameter	Estimate	Std. Error	t-stat	p-value
b	2.130	0.4728	4.505	$6.630e^{-6}$
e	239.6	39.78	6.022	$1.722e^{-9}$

Concentration-Response Model Fit:



A.2.4 Fourth most acutely sensitive genus: *Hyalella* (amphipod)

Hyalella azteca

Prosser et al. (2017a) performed a 96-hour static, measured acute test of N-(1,3-Dimethylbutyl)-N'-phenyl-1,4-phenylenediamine (6PPD, CAS # 793-24-8, >98% purity, obtained from TCI) with the amphipod, *Hyalella azteca*. Juvenile amphipods (7-11 days old) used for testing in the water-only exposure were cultured at the Environment and Climate Change Canada (ECCC), Centre for Inland Waters (Burlington, Canada). Exposure solutions were prepared by spiking dechlorinated City of Burlington tap water with a concentrated solution of 6PPD in acetone as a solvent. Fifteen amphipods were added to 250 mL glass beakers with

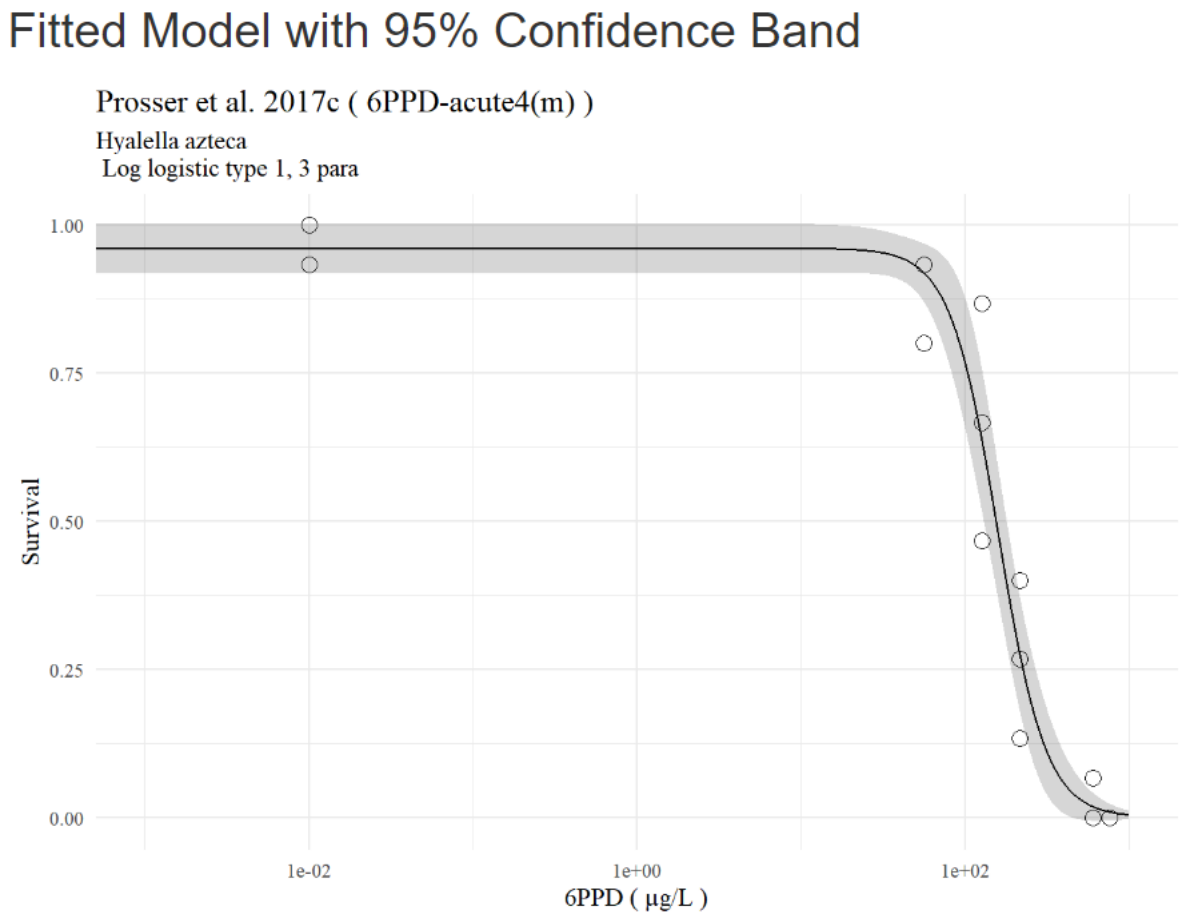
200 mL of test solution and a piece of cotton gauze. Beakers were gently aerated throughout the exposure. Vessels were incubated at 23℃ with a 16 hour light, 8 hour dark photoperiod at a light level of ~200 lux. Treatments included a control and solvent control (<0.1% by volume) and nominal test concentrations of 125, 250, 500, 1,000 and 2,000 µg/L 6PPD. Each treatment was replicated three times. Measured concentrations averaged 22% lower than nominal concentrations overall at test initiation. During testing, pH, dissolved oxygen, conductivity and ammonia ranged from 8.18-8.38, 7.01-9.37 mg/L, 348-394 µS/cm and <0.01-0.025 mg/L, respectively. Only three of the ninety amphipods died in the negative (1 out of 45) and solvent (2 out of 45) controls. Mean measured 6PPD concentrations at test initiation were 15-31% less than nominal concentrations and 75-90% less at test termination. The author-reported 96 hour LC_{50} was 250 µg/L 6PPD, based on initial concentrations. The EPA curve fit the C-R data to calculate a LC_{50} value based on average concentrations instead of initial concentrations. The EPA-calculated LC_{50} was 159.7 µg/L 6PPD, which is acceptable for quantitative use.

A.2.4.1 Prosser et al. (2017a) Concentration-Response Curve – Hyalella azteca (Amphipod)

Publication: Prosser et al. 2017a
Species: *Hyalella azteca*
EPA-Calculated LC_{50}: 159.7 µg/L
Concentration-Response Model Estimates:

Parameter	Estimate	Std. Error	t-stat	p-value
b	2.9796	0.5331	5.590	$2.277e^{-8}$
d	0.9601	0.0212	45.29	$2.2e^{-16}$
e	159.69	1.359	11.95	$2.2e^{-16}$

Concentration-Response Model Fit:



A.2.5 Fifth most acutely sensitive genus: *Daphnia* (cladoceran)

Daphnia magna

Monsanto Co. (1984) performed a 48-hour static, unmeasured acute test of N-(1,3-Dimethylbutyl)-N'-phenyl-1,4-benzenediamine [6PPD, CAS # 793-24-8, purity not reported; assumed to be high purity (> 98%) since the test was part of OECD's 6PPD assessment (OECD 2004a), obtained from P.R. Graham, Monsanto Industrial Chemicals (MIC)] on the cladoceran, *Daphnia magna*. Acute tests were conducted both immediately after introducing the chemical to well water and after the chemicals had been aged for 24 hours. Toxicity tests followed MIC Environmental Assessment Method for Conducting Acute Toxicity Tests with *Daphnia magna*

(Grueber and Adams 1980) and Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians (U.S.EPA 1975). Daphnids used for testing were cultured at the MIC aquatic laboratory and were less than 24 hours old at test initiation. Test concentrations were prepared by dissolving 6PPD in acetone, adding it to the dilution water (well water from St. Peters, MO) and then shaking solutions vigorously for one minute. Ten daphnids were added to 250 mL beakers containing 200 mL of test solution. Test treatments in the unaged study included a well water only (negative) control, a solvent control (1 mL/L acetone; equal to concentration of acetone in the highest test treatment), and five nominal treatment concentrations of 0.25, 0.5, 1.0, 2.0 and 4.0 mg/L, or 250, 500, 1,000, 2,000, and 4,000 µg/L 6PPD, respectively). The aged study included a water-only and solvent control (1 mL/L acetone), plus three nominal treatment concentrations of 0.25, 0.5 and 1.0 mg/L, or 250, 500, and 1,000 µg/L 6PPD. Each treatment was replicated three times for both studies. During testing, pH, dissolved oxygen, alkalinity and hardness ranged from 7.6-8.3, 6.4-8.5 mg/L, 210-290 mg/L and 218-274 mg/L, respectively. The test temperature was 22.0°C. No mortality was observed in the water-only or solvent controls at 48 hours in either study. At 1,000 µg/L 6PPD, 26.7% of the daphnids were immobilized at 24 hours increasing to 100% of the daphnids dead or immobilized at 48 hours. No effects were seen at 250 µg/L 6PPD. The author-reported 48 hour EC_{50} based on immobility was 510 µg/L 6PPD in the unaged study. The EPA did not curve fit the data since this species was not among the most sensitive. The author-reported LC_{50} from the unaged study was adjusted to lower the value by 40% in order to account for loss of 6PPD over experiment duration. The adjusted author-reported LC_{50} was 306.0 µg/L 6PPD and was acceptable for quantitative use. Zero percent mortality was observed in the aged study across all treatments, but since the study is unmeasured the actual test concentrations after aging are unknown, and because of the known instability of

6PPD in water, the study was acceptable for qualitative use only.

Ministry of the Environment, Japan (2019) performed a 48-hour static, measured acute test [based on study details provided in OECD (2004a)] of N-(1,3-dimethylbutyl)-N'-phenyl-1,4-benzenediamine (6PPD, CAS # 793-24-8, purity not reported) on the cladoceran, *Daphnia magna*. The test method used followed OECD TG 202 (Daphnids Acute Immobilization Test) (OECD 2000). No details were provided with regards to source of daphnids, preparation of test solutions, or exposure conditions. Adequate control survival and other test acceptability requirements were assumed per OECD test guidelines. The author-reported 48-hour EC_{50} for the test was 230 µg/L. It was unclear when the test concentration measurements were taken. EPA assumed them to be average concentrations. Therefore, the author-reported value was used. The test result was considered acceptable for quantitative use despite missing study details because the test was conducted by the Ministry of the Environment in Japan and following OECD test guidelines. The deficiencies included information lacking on the chemical purity of 6PPD used in the toxicity test, although high purity (> 98%) was assumed since the test followed OECD test guidelines and was used in OECD's 6PPD assessment (OECD 2004a). Further, it appeared the source of the 6PPD was Bayer AG and OECD (2004a) states "*In Germany 6PPD is manufactured in an industrial scale only at the Bayer AG Brunsbüttel plant. In a continuously working closed system 4-aminodiphenylamine is reacted with an excess of methyl isobutyl ketone (MIBK) to a Schiff´s base. This base is then hydrogenated catalytically. The excess of MIBK is separated off. The hydrogenation by-products are purged with steam. Impurities are removed by distillation under reduced pressure yielding 6PPD with a purity of > 98 %.*"

Hiki et al. (2021) performed a 48-hour static, measured test of N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine (6PPD, CAS# 793-24-8, >98% purity, purchased from Tokyo

Kasei; Tokyo, Japan) with the cladoceran, *Daphnia magna*. The acute toxicity test followed OECD guideline number 202 (OECD 2004b). Neonate (<24 hours old) daphnia used for testing were obtained from brood stock maintained at the National Institute of Environmental Studies. A stock solution was made by dissolving the chemical in dechlorinated tap water with M4 medium (OECD 2004b) and an acetone solvent (assumed based on study details provided by the study author, which also included information on exposures to 6PPD-q). Treatments included a negative control and a single treatment (138 µg/L measured average concentration). The test was conducted in 50 mL glass beakers, each containing 5 daphnids, with four replicate beakers per treatment. Dissolved oxygen, pH, temperature, and conductivity ranged from 7.70-8.54 mg/L, 8.00-8.41, 21.9°C, and 63.7-64.3 mS/m, respectively. The photoperiod was 16 hours light and 8 hours dark. No mortality was observed in the control and 100% mortality was observed at 138 µg/L. The author-reported LC_{50} of <138 µg/L 6PPD was based on time-weighted average concentrations over the 48-hour exposure duration and was considered acceptable for quantitative use.

A.2.6 Sixth most acutely sensitive genus: *Pimephales* (fathead minnow)

Pimephales promelas

Monsanto Co. (1979) performed a 28-day flow-through, measured acute test of N-(1,3-Dimethylbutyl)-N'-phenyl-p-phenylenediamine [6PPD, CAS # 793-24-8, purity not reported; assumed to be high purity (> 98%) since the test was part of OECD's 6PPD assessment (OECD 2004a)] with the fathead minnow, *Pimephales promelas*. Fish (1.3 g, 40.1 mm) used for testing were obtained from the Fender's Fish Hatchery (Brady, NE) and acclimated to test conditions for at least 14 days prior to testing. Stock solutions were made by dissolving the chemical in nanograde acetone. The stock was diluted with aerated well water to make six nominal test concentrations (0, 0.066, 0.12, 0.23, 0.45 and 1.0 mg/L 6PPD). Details about the use of a solvent

control were not provided. Thirty fish were added to 30 L glass aquaria under flow-through conditions delivering solutions at 300 mL/minute/aquarium. Test aquariums were held at 22°C with a 16 hour light, 8 hour dark photoperiod throughout testing. Mean measured concentrations were determined on day 0, 1, 5, 10 ,14, 21 and 28 days with mean measured test treatments of 0.013, 0.024, 0.034, 0.089, 0.26 and 0.92 mg/L, or 13, 24, 34, 89, 260, and 920 µg/L 6PPD, respectively. Average temperature, D.O., pH, and ammonia during the first five days of the 28-day study were 21.5°C, 7.5 mg/L, 7.8, and 0.9 mg/L, respectively. No mortality was observed after 24 hours of exposure across all treatments. At 48 hours, four of 30 fish died in the highest test treatment (1.0 mg/L nominal 6PPD), and at 96 hours 100% mortality was observed in the highest test treatment. The author-reported 96-hour LC_{50} was 450 µg/L 6PPD, based on nominal concentrations. The author-reported LC_{50} value was adjusted by lowering the value by 40% in order to account for loss of 6PPD over the experiment. The adjusted, author-reported LC_{50} was 270.0 µg/L 6PPD, and was acceptable for quantitative use despite the unreported chemical purity for the test compound (6PPD). Furthermore, the reported toxicity value was in line with others reported and this test was used in OECD's 6PPD assessment (OECD 2004a).

A.2.7 Seventh most acutely sensitive genus: *Lampsilis* (freshwater mussel)

Lampsilis fasciola

Prosser et al. (2017b) performed a 48-hour static, measured acute test of N-(1,3-Dimethylbutyl)-N'-phenyl-1,4-phenylenediamine (6PPD, CAS # 793-24-8, >95% purity, obtained from TCI, Portland, OR) with the freshwater bivalve, *Lampsilis fasciola*. Glochidia used for testing in the water-only exposure were isolated from field collected mussels from the Speed River (Ontario, Canada). Glochidia exposures followed ASTM E2455-06 test methodology (ASTM 2013). Viability of field collected glochidia was assessed with NaCl prior to testing to ensure ≥90% viability. Approximately 500-1,000 glochidia were placed in 250 mL

beakers and exposed to one of seven nominal test concentrations 0, 125, 250, 500, 1,000, 2,000 and 4,000 µg/L 6PPD for 48 hours. Each treatment was replicated four times. A volume of acetone representing ≤0.1% of the volume of stock solution was used to introduce 6PPD to moderately hard water to create the different treatments. Per the authors, solvent treatments were not included in the 6PPD experiments because ≤0.1% of acetone by volume had no effect on the viability of glochidia in testing with other toxicants. Subsets of glochidia in control treatments were assessed after 24 and 48 hours for viability and were >90% across replicates at 24 hours (note: the maximum test duration recommendation for glochidia is 24 hours). Vessels were incubated at 20°C with a 16 hour light, 8 hour dark photoperiod. Water chemistry conditions were measured at test initiation and termination and averaged: pH = 8.17, D.O. = 8.26 mg/L, conductivity = 324 µS/cm, chloride = 3.0 mg/L, ammonia <0.01 mg/L, and salinity = 0.16 g/L. Mean measured 6PPD concentrations at test initiation (<0.02, 50.1, 101.5, 168.0, 483.1, 1,238.4, and 3,123.9 µg/L) were 22-66% less than nominal concentrations. There was no information in the publication about time of death. The author-reported 24-hour EC_{50} (based on viability) was 260 µg/L 6PPD. It is unclear if the author-reported EC_{50} was based on measured initial concentrations or nominal concentrations. The EPA did not curve fit the C-R data since this test was outside the most sensitive species. The author-reported EC_{50} value was adjusted to lower the value by 40% in order to account for loss of 6PPD over the experiment. The adjusted, author-reported EC_{50} was 156.0 µg/L 6PPD and was acceptable for quantitative use.

Lampsilis siliquoidea

Prosser et al. (2017b) also performed a 48-hour static, measured acute test of N-(1,3-Dimethylbutyl)-N'-phenyl-1,4-phenylenediamine (6PPD, CAS # 793-24-8, >95% purity, obtained from TCI, Portland, OR) with the fatmucket, *Lampsilis siliquoidea*. Glochidia used for testing in the water-only exposure were isolated from field collected mussels from the Maitland

River (Ontario, Canada). Glochidia exposures followed ASTM E2455-06 test methodology (ASTM 2013). Viability of field collected glochidia was assessed with NaCl prior to testing to ensure ≥90% viability. Approximately 500 - 1,000 glochidia were placed in 250 mL beakers and exposed to one of seven nominal test concentrations 0, 160, 260, 430, 720, 1,200 and 2,000 µg/L 6PPD for 48 hours. Each treatment was replicated four times. A volume of acetone representing ≤0.1% of the volume of stock solution was used to introduce 6PPD to moderately hard water to create the different treatments. Per the authors, solvent treatments were not included in experiments because ≤0.1% of acetone by volume had no effect on the viability of glochidia in testing with other toxicants. Subsets of glochidia in control treatments were assessed after 24 and 48 hours for viability and were >90% across replicates at 24 hours (note: the maximum test duration recommendation for glochidia is 24 hours). Vessels were incubated at 20°C with a 16 hour light, 8 hour dark photoperiod. Water chemistry conditions were measured at test initiation and termination and averaged: pH = 8.28, D.O. = 8.44 mg/L, conductivity = 349 µS/cm, chloride = 1.29 mg/L, ammonia <0.01 mg/L, and salinity = 0.18 g/L. Mean measured 6PPD concentrations at test initiation were 6-21% less than nominal concentrations and 70-94% less at test termination (48 hours). There was no information in the publication about time of death. The 24-hour EC_{50} (based on viability) was 955 µg/L 6PPD. It is unclear if the author-reported EC_{50} was based on measured initial concentrations or nominal concentrations. The EPA did not curve fit the C-R data since this test was outside the most sensitive species. The author-reported EC_{50} value was adjusted to lower the value by 40% in order to account for loss of 6PPD over experiment duration. The adjusted, author-reported EC_{50} was 573.0 µg/L 6PPD and was acceptable for quantitative use.

A.2.8 Eighth most acutely sensitive genus: *Danio* (zebrafish)

Danio rerio

Varshney et al. (2022) performed a 96-hour static-renewal, unmeasured acute test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine (6PPD, CAS # 793-24-8, > 98.0% purity, obtained from CymitQuimica Chemical, Barcelona, Spain) with the zebrafish, *Danio rerio*. AB strain embryos (< 16 cell stage) used for testing came from in-house cultures at the Nord University. Stock solutions (2.5 mg/mL) were made by dissolving the chemical in molecular grade ethanol. Stock solutions were diluted in ISO standard fish media water (ISO 7346-3) to make ten test treatments (0-1,500 µg/L 6PPD). Treatments also included a 0.1% ethanol solvent control. For each treatment one embryo per well was added to clear polystyrene 24-well plates containing 2 mL of test solution (24 embryos per treatment) that had been pre-treated with the respective solutions for 24 hours. Each well plate was covered and incubated at 26±1℃ with a daily cycle of 12 hours light, 12 hours dark. Test solutions were renewed daily. pH averaged 6.81 and oxygen saturation was 87.7% throughout the experiment. All control organisms underwent normal development, with zero mortality. Mortality was observed at 24 hours across 6PPD treatments with approximately 55% mortality observed at 1,200 µg/L. Reported LC_{50} concentrations decreased as exposure length increased: 1,384.93 µg/L 6PPD at 24 hours to 442.62 µg/L 6PPD at 96 hours. The 96 hour LC_{50} was 442.62 µg/L 6PPD, based on nominal concentrations. The author-reported LC_{50} value was adjusted to lower the value by 40% in order to account for loss of 6PPD over the experiment. The adjusted, author-reported LC_{50} was 265.6 µg/L 6PPD, which was acceptable for quantitative use.

Fang et al. (2023) conducted a 94-hour static-renewal, unmeasured acute test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine (6PPD, >98% purity, purchased from Aladdin (Shanghai, China)) with the zebrafish, *Danio rerio*. Wild-type AB line zebrafish [purchased from

the China Zebrafish Resource Center (Wuhan, China)] were cultured in glass tanks, with the water temperature maintained at 28 ± 1°C and a 14:10 hour (light: dark) photoperiod. In addition, the water used in the exposure (pH: 7.0-7.3, $CaCO_3$: 100-200 mg/L, salinity: 0.25-0.75 %, conductivity: 450-550 µS) was aerated. Fish were fed freshly hatched brine shrimp twice daily. To obtain embryos, female and male zebrafish were placed into spawning boxes at night, and the next day, the lights and pumps were turned on and turned off, respectively, at 8 a.m. Only healthy embryos were collected for experiments. The acute toxicity of 6PPD to zebrafish embryos was evaluated following the guidelines issued by the OECD No. 236 (OECD 2013), which began at 2 hpf and ended at 96 hpf. 6PPD was dissolved in acetonitrile and stored at 4°C until used in the experiments. Six nominal concentrations of 6PPD (250, 500, 750, 1,000, 1,250 and 1,500 µg/L) were used in the acute toxicity test. No details were provided if the control treatment represented a solvent or water only (negative) control. Thirty fertilized embryos were placed in a 25 mL glass beaker with 10 mL exposure solution, which were tested in triplicate at each concentration. The exposure solution was replaced every 12 hours. The author-reported 94-hour LC_{50} was 737 µg 6PPD/L, based on nominal concentrations. The author-reported LC_{50} value was adjusted to lower the value by 40% in order to account for loss of 6PPD over the experiment. The adjusted, author-reported LC_{50} was 442.2 µg/L 6PPD, which was acceptable for quantitative use.

Appendix B Quantitative Acute Estuarine/Marine Toxicity Data

There were no quantitatively acceptable empirical data for acute 6PPD toxicity for estuarine/marine species at the time of this literature review (completed in December 2023).

Appendix C Quantitative Chronic Freshwater Toxicity Data

C.1 Summary Table of Acceptable Quantitative Chronic Freshwater 6PPD Toxicity Data

Species (lifestage)	Method[a]	Test Duration	Chemical / Purity	pH	Temp. (°C)	Hardness (mg/L as $CaCO_3$)	Effect	Author Reported Effect Conc. (µg/L)	Species Mean chronic Value (mg/L)	Reference
Fathead minnow, *Pimephales promelas*	F, M	28 d	6PPD Not reported	7.7-7.9	21-22	250	LC_{50}	150	150	Monsanto Co. (1979)
Medaka, *Oryzias latipes*	S, U	ELS (not reported)	6PPD Not reported	-	-	-	NOEC-LOEC (not reported)	3.7-11	6.380	Japan Ministry of the Environment (2019)

a S=Static, R=static-renewal, F=Flow-through, U=unmeasured, M=measured

C.2 Detailed 6PPD Chronic Freshwater Toxicity Study Summaries

This appendix presents detailed study summaries for tests that were considered quantitatively acceptable for chronic screening value derivation. Study summaries are presented below in order of taxonomic sensitivity to 6PPD. The EPA did not develop Concentration-response (C-R) models for these chronic toxicity values as a screening value could not be derived for chronic exposures in freshwaters given the limited data available.

C.2.1 Most chronically sensitive genus: *Oryzias* (Medaka)

Ministry of the Environment, Japan (2019) performed an early-life stage (ELS) test of unknown duration with N-(1,3-dimethylbutyl)-N'-phenyl-1,4-benzenediamine (6PPD, CAS # 793-24-8, purity not reported) on the Japanese medaka, *Oryzias latipes*. This species is not a North American resident species, but *O. latipes* is a common aquatic toxicity test species that serves as a surrogate for untested fish species residing in North America. The toxicity test method used followed OECD TG 210 (Fish Early Life Stage Toxicity Test). No details are provided with regards to source of fish, preparation of test solutions, or exposure conditions. Adequate control survival and other test acceptability requirements were assumed per OECD test guidelines. The reported NOEC and LOEC for the ELS test were 3.7 and 11 µg/L (MATC = 6.380 µg/L). The MATC was considered acceptable for quantitative use.

C.2.2 Second most chronically sensitive genus: *Pimephales* (fathead minnow)

Monsanto Co. (1979) performed a 28-day flow-through, measured chronic test of N-(1,3-Dimethylbutyl)-N'-phenyl-1,4-phenylenediamine [6PPD, CAS # 793-24-8, purity not reported; assumed to be high purity (> 98%) since the test was part of OECD's 6PPD assessment (OECD 2004a)] with juvenile fathead minnow, *Pimephales promelas*. The fathead minnows (1.3 g, 40.1 mm standard length) used in the test were obtained from Fender's Fish Hatchery in Brady, Nebraska. All test fish were held in culture tanks on a l6-hour light, 8 hour dark

photoperiod and acclimated for at least 14 days prior to testing. 6PPD stock solutions were prepared in nanograde acetone. Diluent was aerated well water. Details about the use of a solvent control were not provided. A Mount and Brungs style proportional diluter system was used to deliver five nominal concentrations of 6PPD: 0.066, 0.12, 0.23, 0.45, and 1.0 mg/L and control (well water) to each of the six 30 L glass test aquaria after test solutions had been flowing through the aquaria for 24 hours. Each aquaria held 30 fathead minnows and received control water or test solution at a rate of 300 mL/minute, an amount sufficient to replace the 30 liter test volume at least 14 times in a 24 hour period. The test aquaria were immersed in a circulating water bath held at 22 ± 2°C. The fish were observed for mortality and abnormal behavior initially and once every 24 hours for the remainder of the 28-day test period. The mean measured concentrations of 6PPD were determined on days 0, 1, 5, 10, 14, 21, and 28 and were 0.024, 0.034, 0.089, 0.26 and 0.92 mg/L. Measured concentrations were 92, 58, 39, 28 and 36% of the nominal concentrations progressing from highest to lowest concentration. No mortality was observed after 24 hours of exposure across all treatments. Mortality in 6PPD treatments increased as time progressed, with no mortality observed in the control or 0.066 mg/L 6PPD treatment at test termination. The 28-day LC_{50} based on nominal concentrations was calculated as 150 µg/L 6PPD. The test result was considered acceptable for quantitative use despite the unreported chemical purity for the test compound 6PPD as this test was used in OECD's 6PPD assessment (OECD 2004a).

Appendix D Quantitative Chronic Estuarine/Marine Toxicity Data

There were no quantitatively acceptable empirical data for chronic 6PPD toxicity for estuarine/marine species at the time of this literature review (completed in December 2023).

Appendix E Acute Qualitative Toxicity Data

E.1 Freshwater

Species (lifestage)	Method[a]	Test Duration	Chemical / Purity	pH	Effect	Reported Effect Conc. (µg/L)	Deficiencies	Reference
Cladoceran (<24 hr), *Daphnia magna*	S, U	48 hr	6PPD Not reported	7.6-8.3	EC50 (immobility)	>1,000 (aged for 24 hours)	Only three exposure treatments	Monsanto Co. (1984)
Amphipod (3-5 d), *Hyalella azteca*	R, M	96 hr	6PPD >98%	8.0	100% mortality	<286	Only one exposure concentration	Hiki et al. (2021)
Rainbow trout *Oncorhynchus mykiss*	S, U	96 hr	6PPD Not reported	6.8-7.6	LC_{50}	140	Limited test details; dilution water not reported	Monsanto Co. (1977)
Rainbow trout (juvenile, 2 mo., 0.3-0.7 g), *Oncorhynchus mykiss*	S, M	96 hr	6PPD Not reported	-	LC_{50}	>69.0	Greater than low value when compared to other Quantitative *Oncorhynchus* effect concentrations	Nair et al. (2023)
Zebrafish (embryo, <3 hpf), *Danio rerio*	R, M	96 hr	6PPD >98%	7.7	LC_{50}	>137	Only one exposure concentration	Hiki et al. (2021)
Zebrafish (embryo, 2 hpf), *Danio rerio*	R,U	96 hr	6PPD >99.0%	7	LC_{50}	2,200	Limited test details; number of organisms per replicate uncertain. Use of controls assumed.	Peng et al. (2022)
Zebrafish (adult, 4 mo), *Danio rerio*	S, M	12 hr	6PPD 98.0%	-	LOEC (swimming speed and distance)	1,000	Duration too short; atypical endpoint	Ji et al. (2022)

Species (lifestage)	Method[a]	Test Duration	Chemical / Purity	pH	Effect	Reported Effect Conc. (µg/L)	Deficiencies	Reference
Zebrafish (embryo, 8 hpf), *Danio rerio*	R, U	112 hr	6PPD >98%	-	NOEC (mortality)	1,200	Atypical test duration (too long for an acute test)	Zhang et al. (2023)
Bluegill, *Lepomis macrochirus*	S, U	96 hr	6PPD Not reported	6.5-7.2	LC_{50}	400	Limited test details; dilution water not reported	Monsanto Co. (1977)
Medaka (41 d), *Oryzias latipes*	R, M	96 hr	6PPD >98%	7.9	80% mortality	<107	Only one exposure concentration	Hiki et al. (2021)

a S=Static, R=static-renewal, F=Flow-through, U=unmeasured, M=measured

E.1.1 Detailed Study Summaries of Acute Qualitative Data

E.1.1.1 Oryzias latipes

Hiki et al. (2021) performed a 96-hour static-renewal, measured test of N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine (6PPD, CAS# 793-24-8, >98% purity, purchased from Tokyo Kasei; Tokyo, Japan) with the Japanese medaka, *Oryzias latipes*. The acute toxicity test followed OECD guideline number 203 (OECD 2019). Immature (41 day old) fish used for testing were obtained from brood stock maintained at the National Institute of Environmental Studies. A stock solution was made by dissolving the chemical in dechlorinated tap water with acetone. The test solutions were renewed (>90% renewal) after 48 hours. Treatments included a negative control and a single treatment (107 µg/L measured average concentration). The test was conducted in 5 L glass aquaria. Ten fish were added to each aquarium, with one aquarium each for the negative control and treatment. Dissolved oxygen, pH, temperature, and conductivity averaged 8.05 mg/L, 7.9, 24.4 ℃, and 33.8 mS/m, respectively. The photoperiod was 16 hours light and 8 hours dark. No mortality was observed in the control and 80% mortality was observed in the 6PPD treatment, resulting in a 96-hour LC_{50} of <107 µg/L. The value was considered acceptable for qualitative use because there was only one test concentration, and the resulting concentration was not definitive.

E.1.1.2 Hyalella azteca

Hiki et al. (2021) performed a 96-hour static-renewal, measured test of N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine (6PPD, CAS# 793-24-8, >98% purity, purchased from Tokyo Kasei; Tokyo, Japan) with the amphipod, *Hyalella azteca*. The acute toxicity test followed the test method outlined by Environment and Climate Change Canada (2017)(ECCC 2017). Neonate (3-5 day old) amphipods used for testing were obtained from brood stock maintained at the National Institute of Environmental Studies. A stock solution was made by

dissolving the chemical in dechlorinated tap water with acetone. The test solutions were renewed (>90% renewal) after 48 hours. Treatments included a negative control and a single treatment (286 µg/L time-weighted measured average concentration). The test was conducted in 300 mL glass beakers, each containing 10 amphipods, with two replicate beakers per treatment. Test organisms were fed at the start of the experiment and again after 48 hours. Dissolved oxygen, pH, temperature, and conductivity averaged 8.05 mg/L, 8.0, 23.6 °C, and 31.1 mS/m, respectively. The photoperiod was 16 hours light and 8 hours dark. Mortality in the control was 5%, and mortality in the single test concentration was 100%. The 96-hour LC_{50} was <286 µg/L. The value was considered acceptable for qualitative use only because there was only one test concentration, and the effect concentration was not definitive.

E.1.1.3 Daphnia magna

Monsanto Co. (1984) performed a 48-hour static, unmeasured acute test of N-(1,3-dimethylbutyl)-N'-phenyl-1,4-benzenediamine [6PPD, CAS # 793-24-8, purity not reported; assumed to be high purity (> 98%) since the test was part of OECD's 6PPD assessment (OECD 2004a)] on the cladoceran, *Daphnia magna*. Acute tests were conducted immediately after introducing the chemical to well water and after the 6PPD in solution had been aged for 24 hours. Toxicity tests followed MIC Environmental Services, *Environmental Assessment Method for Conducting Acute Toxicity Tests with Daphnia magna* (Grueber and Adams 1980) and *Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians* (U.S.EPA 1975). Daphnids used for testing were cultured at the MIC Environmental Services aquatic laboratory and were less than 24 hours old at test initiation. Test concentrations were prepared by dissolving 6PPD in acetone, adding it to the dilution water (well water from St. Peters, MO) and then shaking solutions vigorously for one minute. Ten daphnids were added to 250 mL beakers

containing 200 mL of test solution. Test treatments included a well water only (negative control), a solvent control (1 mL/L; equal to concentration of acetone in the highest test treatment), and 0.25, 0.5, 1.0, 2.0 and 4.0 mg/L 6PPD treatments in the unaged study, and negative control, solvent control, and 0.25, 0.5 and 1.0 mg/L treatments in the aged study. Each treatment was replicated three times. During testing pH, dissolved oxygen, alkalinity and hardness ranged from 7.6-8.3, 6.4-8.5 mg/L, 210-290 mg/L and 218-274 mg/L, respectively. The test temperature was 22.0°C. No mortality was observed in the negative or solvent controls at 48 hours in either exposure. The 48-hour EC_{50} based on immobility was 510 µg/L 6PPD in the unaged study and was acceptable for quantitative use. Zero percent mortality was observed across all treatments in the aged study (i.e., no effect up to the nominal concentration of 1,000 µg/L 6PPD), but since the study was unmeasured and the half-life of 6PPD is less than 24 hours the actual test concentrations are unknown, and therefore, the test result for this study was considered acceptable for qualitative use only at this time.

E.1.1.4 Danio rerio

Hiki et al. (2021) performed a 96-hour static-renewal, measured test of N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine (6PPD, CAS# 793-24-8, >98% purity, purchased from Tokyo Kasei; Tokyo, Japan) with the zebrafish, *Danio rerio*. The acute toxicity test followed OECD guideline number 236 (OECD 2013). Embryos (16 cell stage) used for testing were obtained from brood stock maintained at the National Institute of Environmental Studies. A stock solution was made by dissolving the chemical in dechlorinated tap water with acetone. The test solutions were renewed (>90% renewal) after 48 hours. Treatments included a negative control and a single treatment (137 µg/L measured time-weighted average concentration). The test was conducted in 24-well plates, with 2 mL test solution added per plate. One embryo was

added to each well, for a total of 20 embryos each for the negative control and treatment, respectively. Dissolved oxygen, pH, temperature, and conductivity averaged 8.0 mg/L, 7.7, 25.9°C, and 31.6 mS/m, respectively. The photoperiod was 16 hours light and 8 hours dark. No mortality was observed in the control or test concentration resulting in a 96-hour LC_{50} of >137 µg/L. This value was considered acceptable for qualitative use because there was only one test concentration, and the value was not definitive.

Ji et al. (2022) performed a 12-hour static, measured test of N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine (6PPD, CAS# 793-24-8, 98.0% purity, obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China)) with the zebrafish, *Danio rerio*. Adult (4 month old, 0.30±0.05g wet weight) AB strain zebrafish were purchased from the Beijing Hongda Gaofeng Aquarium Department and were acclimated in fish facility (control) water for 14 days prior to the experiment. A 50,000 mg/L stock solution of 6PPD was prepared in acetone. Stock solution was added to aerated water to create three nominal treatment levels (50, 500, and 1,000 µg/L 6PPD, respectively), so that every treatment had an acetone concentration of 200 µL/L. The test was conducted in acrylic tanks (30 cm x 30 cm x 18 cm), subdivided into four compartments. Water was added so that each compartment included 1 L of water. Twelve fish were added to each treatment level (plus a water-only control), at a density of one fish per liter of water, so that the control and each treatment consisted of three replicate acrylic tanks. Fish were not fed for 24 hours prior to the experiment or during the experiment. The test water during the experiment was 26°C. Swimming velocity decreased by 42.4% at 1,000 µg/L when compared to the control, which was statistically significant, resulting in a 12-hour LOEC of 1,000 µg/L 6PPD. The test was considered qualitative because it was of insufficient duration for an acute toxicity test and was based on a non-apical behavioral endpoint.

Peng et al. (2022) performed a 96-hour static-renewal, unmeasured test of N-(1,3-dimethylbutyl)-N′ -phenyl-p-phenylenediamine [6PPD, CAS# 793-24-8, >99.0% purity, obtained from Sinopharm Chemical Reagents Co., Ltd. (Shanghai, China)] with the zebrafish, *Danio rerio*. Wild-type (AB strain) zebrafish embryos two hours post fertilization (hpf) purchased from the Institute of Hydrobiology of the Chinese Academy of Science were used for the experiments. A stock solution of 6PPD at a concentration of 1000 mg/L was prepared by dissolving in dimethyl sulfoxide (DMSO; purity>99%). The test was carried out according to Organization for Economic Co-operation and Development (OECD) test guideline 236 (fish embryo toxicity test). Nominal exposure concentrations (5, 2.5, 1.25, 0.625, and 0.3125 mg/L 6PPD) were determined based on the results of experiments. Each treatment consisted of three replicates, and the DMSO concentration in all groups was less than 0.001%. Embryos (2 hpf) were randomly sorted into 24-well plates for 96 h exposure to 6PPD. Each well contained 2 mL of exposure solution and one embryo. The controlled experimental conditions were 28 ± 0.5°C with a 14:10 h light-dark photoperiod cycle. The dead embryos were identified by egg coagulation, unformed body segments, unseparated tails, and no heartbeat. These embryos were counted and removed every day. During the experimental period, the exposure solutions were renewed every 24 hours. The 96-hour LC_{50} was calculated as 2,200 µg/L 6PPD. The test was considered qualitative because of insufficient information regarding organism number, replication, and uncertainty regarding the use of controls and control response.

Zhang et al. (2023) conducted a 112-hour static-renewal, unmeasured acute test of N-(1,3-Dimethylbutyl)-N′-phenyl-p-phenylenediamine [6PPD, >98% purity, purchased from the Tanmo Quality Control Technology Co., LTD (Changzhou, China)] with the zebrafish, *Danio rerio*. Wild-type AB adult zebrafish were housed at Wenzhou Medical University and kept at

standard laboratory conditions of 28℃ on a 14:10 hour (dark: light) photoperiod in a recirculation system. Water supplied to the system was filtered by reverse osmosis (pH 7.0–7.5), and Instant Ocean® salt (Saltwater Aquarium Fish Supplies) was added to raise the water conductivity to 450-1,000 µS/cm (system water). The adult fish were fed twice daily with a zebrafish diet (Zeigler, Aquatic Habitats, Apopka, FL) and live *Artemia* (Jiahong Feed Co., Tianjin, China). Zebrafish embryos were raised in an embryo medium, and embryonic and larvae developmental progression was inspected under a dissecting microscope. Stock solutions (1.2 mg/mL) were prepared by dissolving 6PPD in 100% dimethyl sulfoxide (DMSO) and stored at -20℃. A working solution was prepared by diluting the stock solution immediately before experimental use. A serial dilution series was used with a final DMSO concentration of 0.1%. The negative control animals also received 0.1% DMSO (v/v in embryo medium). Zebrafish embryos at 8 hpf were continuously exposed to 6PPD during the embryo development stage until 120 hpf, with solutions changed at 60 hpf. Twelve-well plates were used to hold 20 embryos and 3 mL exposure solution per well. There were 20 embryos per replicate and three biological triplicates per group. The exposure plate was covered with foil to avoid photolysis. The malformed representative larvae were imaged at 120 hpf. The embryo hatching rate at 48 and 72 hpf, and accumulated malformation and mortality at 120 hpf were recorded. Between 24 and 120 hpf, embryo development was recorded daily under the dissecting microscope, and the malformations such as pericardial edema, yolk sac edema, uninflated swim bladder, eye and pigment abnormity were counted. At 120 hpf, the accumulated malformation and mortality was calculated using triplicates. The author-reported 112-hour mortality NOEC was 1,200 µg 6PPD/L, which was acceptable for qualitative use due to atypical test duration.

E.1.1.5 Oncorhynchus mykiss

Monsanto Co. (1977) performed a 96-hour static, unmeasured acute test of Santoflex 13 [N-(1,3-dimethylbutyl)-N'-phenyl-1,4-benzenediamine [6PPD, CAS # 793-24-8, purity not reported; assumed to be high purity (> 98%) since the test was part of OECD's 6PPD assessment (OECD 2004a)] on rainbow trout, *Oncorhynchus mykiss*. Acute tests were conducted following an in-house protocol. 6PPD, in reagent-grade acetone, was introduced into 15 L of unspecified diluent water in all glass vessels of unknown size. Nominal test concentrations included diluent water (negative control), acetone (solvent control; concentration not reported), 0.087, 0.10, 0.12, 0.14, 0.16, 0.18, 0.24, and 0.42 mg/L 6PPD. Ten juvenile (3.7 cm standard length) rainbow trout were added to each test vessel. Fish were not fed 48 hours prior to testing or during the exposure. Observations and mortality counts were made every 24 hours. No aeration was provided. Test temperature was maintained at 12 ± 1°C. Dissolved oxygen ranged from 9.9 mg/L (93% of saturation) to 2.8 mg/L (26% of saturation) at the beginning and end of exposure, respectively. pH values ranged from 6.8 to 7.6. Mortality was observed after 24 hours in the three highest test concentrations, ranging from 30-100%. After 96 hours mortality was ≥10% across all 6PPD test concentrations except 0.087 mg/L, where no mortality was observed. No mortality was observed in negative or solvent controls. The 96-hour LC_{50} was calculated as 140 µg/L 6PPD. Since the dilution water was not reported and dissolved oxygen decreased to unacceptable levels in the test, the result was considered qualitative.

Nair et al. (2023) conducted a 96-hour measured, static acute test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine [6PPD, purity not provided, purchased from the Toronto Research Chemicals (Toronto, ON, Canada)] with the rainbow trout, *Oncorhynchus mykiss*. Stock solutions used for fish exposure experiments were prepared in HPLC-grade methanol. Rainbow trout eggs were purchased from Lyndon Hatcheries (New Dundee, ON,

Canada). Fish were reared from eggs and cultured under flow-through conditions at 15 ±1°C for six weeks prior to exposure experiments. Fish were monitored daily and fed with a commercial fish feed at a daily rate of 1% of body weight. The acute toxicity test was conducted using juvenile rainbow trout (2 months old, 0.3 – 0.7g), in 20 L plastic containers lined with food grade polyethylene disposable liners at 15 ± 1°C for 96 ± 2 hours. Rainbow trout were exposed to six nominal concentrations (0.2, 0.8, 3, 12 and 50 µg/L) of 6PPD by spiking ~2 mL of methanol stock solution into 20 L of water. The reported measured concentrations were 0.53, 1.15, 3.48, 16.6 and 69.0 µg/L 6PPD. Three replicates were performed for each treatment group, with 10 fish being included in each replicate. Solvent control exposures were dosed with the methanol solvent vehicle at the same level as that of the treatment groups (0.01%). Tests were conducted in static conditions and fish were not fed for at least 16 hours before testing or during exposure. Mortality and immobility of fish were recorded daily. Water samples taken during the exposure were mixed with 0.5 mL of methanol and stored at -80°C until analysis via ultra-high-performance liquid chromatography - mass spectrometry (LC-HRMS). The author-reported 96-hour LC_{50} was >69.0 µg 6PPD/L and was acceptable for qualitative use. The value was excluded from quantitative studies because it was a greater than low value compared to the other quantitative *Oncorhynchus* effect concentration for coho salmon.

E.1.1.6 Lepomis macrochirus

Monsanto Co. (1977) performed a 96-hour static, unmeasured acute test of Santoflex 13 [N-(1,3-dimethylbutyl)-N'-phenyl-1,4-benzenediamine [6PPD, CAS # 793-24-8 purity not reported; assumed to be high purity (> 98%) since the test was part of OECD's 6PPD assessment (OECD 2004a)] on bluegill sunfish, *Lepomis macrochirus*. Acute tests were conducted following an in-house protocol. 6PPD, in reagent-grade acetone, was introduced into 15 L of unspecified

diluent water in all glass vessels of unknown size. Nominal test concentrations included diluent water (negative control), acetone (solvent control; concentration not reported), 0.24, 0.32, 0.42, 0.65, and 1.0 mg/L 6PPD. Ten juvenile (3.8 cm standard length) bluegill were added to each test vessel. Fish were not fed for 48 hours prior to testing or during the exposure. Observations and mortality counts were made every 24 hours. No aeration was provided. Test temperature was maintained at 22 ± 1°C. Dissolved oxygen ranged from 8.6 mg/L (98% of saturation) to 0.2 mg/L (2% of saturation) at the beginning and end of exposure, respectively. pH values ranged from 6.5 to 7.2. Mortality was observed after 24 hours in the two highest test concentrations, ranging from 30-100%. At 96 hours mortality was ≥10% across all 6PPD test concentrations except 0.24 mg/L, where no mortality was observed. No mortality was observed in negative or solvent controls. The 96-hour LC_{50} was reported as 400 µg/L 6PPD. Since the dilution water was not reported and dissolved oxygen decreased to unacceptable levels in the test, the result was considered qualitative.

E.2 Estuarine/Marine

There were no qualitatively acceptable empirical data for acute 6PPD toxicity for estuarine/marine species at the time of this literature review (completed in December 2023).

Appendix F Chronic Qualitative Toxicity Data

F.1 Freshwater Water Only Exposures

There are no qualitatively acceptable empirical data for chronic 6PPD toxicity for freshwater species at the time of this literature review (completed in December 2023).

F.2 Freshwater Sediment Exposures (typically used for criteria development)

Species (lifestage)	Method[a]	Test Duration	Chemical / Purity	pH	Effect	Chronic Limits (NOEC-LOEC) (µg/L)	Reported Effect Conc. (µg/L)[b]	Deficiencies	Reference
Tubificid worm (adult), *Tubifex tubifex*	S, M (sediment)	28 d	6PPD >98.0%	8.28-8.58	EC10 (total juveniles)	-	3 (µg/g dw sediment)	Sediment exposure	Prosser et al. (2017a)
Fatmucket (glochidia), *Lampsilis siliquoidea*	S, M (sediment)	28 d	6PPD >95%	8.03-8.46	LC10	-	5	Sediment exposure	Prosser et al. (2017b)
Amphipod (juvenile, 7-11 d), *Hyalella azteca*	S, M (sediment)	28 d	6PPD >98.0%	8.30-8.51	LC10	-	6	Sediment exposure	Prosser et al. (2017a)
Fathead minnow (egg, <18 hpf), *Pimephales promelas*	S, M (sediment)	21 d	6PPD >98.0%	8.35-8.40	LC_{25}	-	26	Sediment exposure with overlying water; only 21 day duration	Prosser et al. (2017c)

a S=Static, R=static-renewal, F=Flow-through, U=unmeasured, M=measured

b Concentrations reported as µg/L unless noted otherwise.

F.2.1 Study Summaries of Sediment Exposures

F.2.1.1 Pimephales promelas

Prosser et al. (2017c) performed a 21-day static, measured chronic test of N-(1,3-Dimethylbutyl)-N'-phenyl-1,4-phenylenediamine (6PPD, CAS # 793-24-8, 95% purity, obtained from TCI, Portland, OR) with the fathead minnow, *Pimephales promelas* in sediment. Embryos (<18 hpf) used for the sediment exposure were purchased from Aquatox Laboratories (Guelph, ON, Canada). The sediment used was a mixture of two reference sediments (from Long Point Marsh and Long Point Bay in Lake Erie; 2:3 ratio Marsh:Bay with organic carbon content of ˜2%). This mixture of sediments was routinely used for culturing and testing. Stock spiking solutions of 6PPD were produced by dissolving solid 6PPD in acetone. The volume of stock spiking solution added to sediment was <1% by volume. Sediment was spiked in 1-L amber glass jars and mixed for 24 h at 22 ± 2°C. Acetone was evaporated in a fume hood for 48 h and distilled water added to account for any evaporation before sealing the jars and storing at 4 ±2°C for 28 days to allow the 6PPD to equilibrate between the sediment and water. After equilibration, 35 g wet weight of sediment was placed in test vessels (1-L glass beaker) spiked with varying concentrations of 6PPD and 700 mL of overlying culture water. The exposure was renewed daily by transferring eggs or hatched larvae to a newly prepared test vessel. Three test vessels were prepared for each concentration. Twenty embryos in egg cups were placed in each test vessel. Embryos were exposed to one of five nominal test concentrations including a negative solvent control (0), 125, 250, 500, and 1,000 µg/g dw 6PPD. The test vessels were gently aerated and maintained in an incubator at 23.6 ±0.5°C for 28 d. Egg cups were replaced at hatch on day 5, and the number of larvae was reduced to 10 on day 14 (i.e., 9 dph). The exposure ended at 16 dph, which was at day 21 of the test. Because test vessels were replaced every 24 h, water samples were taken [on day 3 (egg stage), day 10 (early larval stage), and day 17 (late larval

stage)] before and after the addition of eggs and larvae to characterize the change in concentration of 6PPD in overlying water over the 24-h period. Each day larvae were fed 10 µL of a solution containing newly hatched brine shrimp (˜15 nauplii/L) per fish in the first week of the larval stage (0–8 dph) and 20 µL in the second week (9–16 dph). Embryos and larvae were inspected daily for deformities or mortality. Mean water chemistry conditions during the test ranged from: pH 8.35-8.42, D.O. 7.37-8.10 mg/L, conductivity 342-379 µS/cm, and ammonia 0.00-0.02 mg/L. Mean measured 6PPD concentrations in overlying water at test initiation test were <0.02 (negative and solvent control), 17.35, 39.24, 83.32, and 192.86 µg/L 6PPD. Between 39 and 65% of the initial concentration of 6PPD remained during the egg state (Days 0-3), and between 43 and 89% remained at test termination. The 28-day LC_{25} based on the concentration of 6PPD in overlying water was 26 µg/L 6PPD. The study result was acceptable for qualitative use because the exposure involved sediment.

F.2.1.2 Tubifex tubifex

Prosser et al. (2017a) performed a 28-day static, measured sediment test of N-(1,3-Dimethylbutyl)-N'-phenyl-1,4-phenylenediamine (6PPD, CAS # 793-24-8, >98.0% purity, obtained from TCI) with the oligochaete, *Tubifex tubifex*. Tubifex used in this study were obtained from a permanent culture maintained at Environment and Climate Change Canada's, Centre for Inland Waters in Burlington, Ontario, Canada. The sediment used was a mixture of two reference sediments (from Long Point Marsh and Long Point Bay in Lake Erie; 2:3 ratio Marsh:Bay with organic carbon content of ˜2%). This mixture of sediments was routinely used for culturing and testing. Stock spiking solutions of 6PPD were produced by dissolving solid 6PPD in acetone. The volume of stock spiking solution added to sediment was <1% by volume. Sediment was spiked in 1-L amber glass jars and mixed for 24 hours at 22 ±2°C. Acetone was

evaporated in a fume hood for 48 hours and distilled water added to account for any evaporation before sealing the jars and storing at 4 ±2°C for three weeks to allow the 6PPD to equilibrate between the sediment and water. After equilibration, 100 mL of sediment was placed in test vessels (1 L glass jar) spiked with varying concentrations of 6PPD and 750 ml of overlying culture water. Overlying water was not changed over the course of the test. Three replicate test vessels were prepared for each concentration. Four worms were placed in each test vessel after the vessels had been aerated for 7 days. Animals were exposed to one of seven nominal test concentrations including a negative control (0), solvent control (0), 2, 20, 100, 200, and 500 µg 6PPD/g dw sediment. The test vessels were gently aerated and maintained in a dark growth chamber at 23 ±2°C for 28 days. Water and sediment were sampled after 7 d of aeration in one replicate without worms to confirm the concentration of 6PPD in the overlying water and sediment at the initiation of the test. Water and sediment were sampled from each replicate for the analysis of 6PPD at the conclusion of the test. Worms were not fed during the exposure. At test termination after 28 days, the sediment in each test vessel was passed through a 500-mm and 250-mm sieve sequentially in order to remove adult worms, juvenile worms, and cocoons. Worms and cocoons were transferred from each sieve to separate Petri dishes and observed under a dissecting microscope. Adult worms were counted, and observations were made on visibility of gonads and overall health of the worms. Mean measured 6PPD concentrations in overlying water at test initiation test were <0.01 (negative and solvent control), <0.01, 0.45, 3.71, 5.42, and 17.8 µg/L 6PPD. Between 91 and 100% of the initial concentration of 6PPD was lost by Day 28. The 28-day EC_{10} based on total juveniles was 3 µg 6PPD/g dw sediment. The study result was acceptable for qualitative use because the exposure involved sediment.

F.2.1.3 Lampsilis siliquoidea

Prosser et al. (2017b) performed a 28-day static, measured chronic test of N-(1,3-Dimethylbutyl)-N'-phenyl-1,4-phenylenediamine (6PPD, CAS # 793-24-8, 95% purity, obtained from TCI, Portland, OR) with the fatmucket, *Lampsilis siliquoidea* in sediment. Juveniles used for the sediment exposure were from laboratory cultures of wild mussels propagated via host-fish infection at Missouri State University. The sediment used was a mixture of two reference sediments (from Long Point Marsh and Long Point Bay in Lake Erie; 2:3 ratio Marsh:Bay with organic carbon content of ˜2%). This mixture of sediments was routinely used for culturing and testing. Stock spiking solutions of 6PPD were produced by dissolving solid 6PPD in acetone. The volume of stock spiking solution added to sediment was <1% by volume. Sediment was spiked in 1-L amber glass jars and mixed for 24 h at 22 ±2°C. Acetone was evaporated in a fume hood for 48 h and distilled water added to account for any evaporation before sealing the jars and storing at 4 ±2°C for 18 d to allow the 6PPD to equilibrate between the sediment and water. After equilibration, 100 mL of sediment was placed in test vessels (1-L glass beaker), followed by the addition of 700 mL of overlying culture water. Five test vessels were prepared for each concentration. Ten juvenile mussels were placed on the surface of the sediment in each test vessel and exposed to one of seven nominal test concentrations including a negative control (0), solvent control (0), 100, 400, 800, 1,600, and 2,000 µg 6PPD/g dw sediment. The test vessels were gently aerated and maintained in an incubator at 20 ± 1°C with a photoperiod of 16 hour light: 8 hour dark (~200 lux) for 28 d. Water and sediment were sampled after 7 days of aeration in one replicate without mussels to confirm the concentration of 6PPD in the overlying water and sediment at the initiation of the test. Mussels were transferred to new test vessels after 14 days of exposure. Water and sediment were sampled from each replicate for the analysis of 6PPD at the conclusion of the test. Juvenile mussels in each vessel were fed 200 mL of food solution twice

daily. At test termination, juvenile mussels from each vessel were moved to clean water and placed in a petri dish containing freshwater aquarium sand for a 3-day period to determine survival or mortality using the burial assay with observation of filtering. Water chemistry conditions were measured at test initiation and termination and averaged: pH 8.37, D.O. 8.33 mg/L, conductivity 474 µS/cm, and ammonia 0.02 mg/L. Mean measured 6PPD concentrations in overlying water at test initiation test were <0.02 (negative and solvent control), 2.34, 10.29, 36.50, 64.29, and 82.88 µg/L 6PPD. Between 90 and 100% of the initial concentration of 6PPD was lost before vessel change on Day 14, and between 71 and 100 % was lost at test termination on Day 28. There was no information in the publication about time of death. The 28-day LC_{10} was 5 µg/L 6PPD. The study result was acceptable for qualitative use because the exposure involved sediment.

F.2.1.4 Hyalella azteca

Prosser et al. (2017a) performed a 28-day static, measured chronic test of N-(1,3-Dimethylbutyl)-N'-phenyl-1,4-phenylenediamine (6PPD, CAS # 793-24-8, 95% purity, obtained from TCI, Portland, OR) with the amphipod, *Hyalella azteca* in sediment. Juvenile amphipods (age 7-11 d) used for the sediment exposure were cultured at Environment and Climate Change Canada's, Centre for Inland Waters in Burlington, Ontario, Canada. The sediment used was a mixture of two reference sediments (from Long Point Marsh and Long Point Bay in Lake Erie; 2:3 ratio Marsh:Bay with organic carbon content of ˜2%). This mixture of sediments was routinely used for culturing and testing. Stock spiking solutions of 6PPD were produced by dissolving solid 6PPD in acetone. The volume of stock spiking solution added to sediment was <1% by volume. Sediment was spiked in-L amber glass jars and mixed for 24 hours at 22 ±2°C. Acetone was evaporated in a fume hood for 48 h and distilled water added to account for any

evaporation before sealing the jars and storing at 4 ±2°C for three weeks to allow the 6PPD to equilibrate between the sediment and water. After equilibration, 50 mL of sediment was placed in test vessels (600 ml glass beaker) spiked with varying concentrations of 6PPD and 350 ml of overlying culture water. Overlying water was not changed over the course of the test. Seven test vessels were prepared for each concentration. Fifteen juvenile amphipods were placed in each test vessel after the vessels had been aerated for 7 days. Animals were exposed to one of seven nominal test concentrations including a negative control (0), solvent control (0), 20, 200, 500, 1,000, and 2,000 µg 6PPD/g dw sediment. The test vessels were gently aerated and maintained in an incubator at 23 ±2°C with a photoperiod of 16 hour light: 8 hour dark (~200 lux) for 28 days. Water and sediment were sampled after 7 days of aeration in one replicate without amphipods to confirm the concentration of 6PPD in the overlying water and sediment at the initiation of the test. Water and sediment were sampled from each replicate for the analysis of 6PPD at the conclusion of the test. Each test vessel received 2.5 mg of ground TetraMin® fish food flakes twice a week in the first two weeks of the test, 2.5 mg of food three times in the third week, and 5 mg of food three times in the final week. At test termination after 28 days, surviving juvenile amphipods from six replicate vessels per treatment were counted and dried to determine growth and production of biomass for each replicate. Water chemistry conditions were measured at test initiation and termination and averaged: pH 8.31, D.O. 8.49 mg/L, conductivity 352 µS/cm, and ammonia 0.004 mg/L. Mean measured 6PPD concentrations in overlying water at test initiation test were <0.01 (negative and solvent control), 1.33, 8.09, 16.2, 28.1, and 40.0 µg/L 6PPD. Between 43 and 100% of the initial concentration of 6PPD was lost by Day 28. The 28-day LC_{10} was 6 µg/L 6PPD. The study result was acceptable for qualitative use because the exposure involved sediment.

F.3 Estuarine/Marine

There were no qualitatively acceptable empirical data for chronic 6PPD toxicity for estuarine/marine species at the time of this literature review (completed in December 2023).

Appendix G Unused Toxicity Data

There were no unused empirical data for acute and chronic 6PPD toxicity for freshwater and estuarine/marine species at the time of this literature review (completed in December 2023).