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Acute Aquatic Life Screening Value for 6PPD-quinone in Freshwater

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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
ATRF	Aquatic Toxicology Research Facility
AWQC	Ambient Water Quality Criteria
CAS	Chemical Abstract Services
CMC	criterion maximum concentration
C-R	concentration-response
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
OW	Office of Water
PPD	para-phenylenediamine
PSCE	Pacific Science Enterprise Centre
SMAV	species mean acute value
SMCV	species mean chronic value
SOP	Standard Operating Procedure
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 quinone (6PPD-quinone, or 6PPD-q). 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-q protective of aquatic life, including sensitive salmonids, 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-q it presents a regulation itself. Thus, this document does not establish or affect legal rights or obligation, 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-q 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
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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 salmonids, from the acute effects of N-(1,3-Dimethylbutyl)-N′-phenyl-p-phenylenediamine quinone (6PPD-quinone or 6PPD-q). This work was undertaken to fulfill a pressing need to establish protective values for 6PPD-q which has been found to be extremely toxic to certain sensitive aquatic species, including sensitive salmonids. 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-q to aquatic organisms.

6PPD-q is a breakdown product of the rubber-tire anti-oxidant and anti-ozonant compound N-(1,3-Dimethylbutyl)-N′-phenyl-p-phenylenediamine (6PPD). It is formed by reaction of 6PPD with ozone and was identified as the likely causative agent for "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-q do not fulfill the EPA's data requirements for deriving national recommended AWQC according to the 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-q and do not fulfill the minimum data requirements (MDRs) described in the EPA's *Guidelines*. In particular, the data on chronic 6PPD-q toxicity and on 6PPD-q toxicity in estuarine/marine waters are 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. Acute toxicity data (quantitative and qualitative) for 6PPD-q were available for seven 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 researchers' consideration of the rapid onset of mortality upon exposure to 6PPD-q, as outlined in the document below. Additionally, when measured over the test duration toxicity tests consistently observed a loss of 6PPD-q, 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-q screening value for aquatic life generally following the derivation methods and calculation approach described in the EPA's *Guidelines*. The acute 6PPD-q 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 the EPA's response to peer review comments (https://www.epa.gov/wqc/acute-6ppd-q-aquatic-life-screening-value-freshwater). The acute screening value concentration is expected to be generally protective of 95% of freshwater species exposed to 6PPD-q 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* and the EPA's 850 Ecological Effects Test Guidelines (or other similar well-accepted test methods)). The science and understanding of 6PPD-q are relatively recent (with 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-q 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-q through the December 2023 quarterly update of the ECOTOXicology database (ECOTOX; https://cfpub.epa.gov/ecotox/) and provides an acute screening value for 6PPD-q 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).

The screening value for acute exposures to 6PPD-q is 11 ng/L (0.011 µg/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 salmonid species, from acute exposures to 6PPD-q. 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 (CWA) for acute exposures to 6PPD-q, 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-phenylenediamine quinone (6PPD-q), 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,"* or *Guidelines* (U.S.EPA 1985). In particular, the data on the chronic 6PPD-q toxicity and on 6PPD-q 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 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 researchers'

consideration of the rapid onset of mortality upon exposure to 6PPD-q, as described below (Section 2.2.2.4). Additionally, when measured over the test duration, toxicity tests consistently indicated a loss of 6PPD-q, 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-q, through the December 2023 quarterly update of ECOTOX. It quantifies the toxicity of 6PPD-q to aquatic organisms, including sensitive salmonids, and provides a screening value to protect aquatic life in freshwater from the acute toxic effects of 6PPD-q.

The EPA derived the screening value for acute exposures to 6PPD-q in freshwaters using the best available data to reflect the latest scientific knowledge on the toxicological effects of 6PPD-q 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-q 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-q-aquatic-life-screening-value-freshwater).

From the limited amount of available data, the 6PPD-q screening value in this document is expected to be protective of the sensitive organisms in freshwater aquatic communities, particularly the coho salmon (*Oncorhynchus kisutch*) which appears to be exceptionally sensitive to 6PPD-q. The freshwater screening value is the EPA's current best estimate of the maximum concentration of 6PPD-q 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, including those observed in sensitive salmonid species such as Coho salmon (*Oncorhynchus kisutch*). 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-q 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-quinone in the Aquatic Environment

6PPD is an anti-ozonant and anti-oxidant additive to vehicle tire rubber, from reactions with ozone and oxygen, which can lead to degradation and cracking (Rossomme et al. 2023; Seiwert et al. 2022; Unice et al. 2015). Because of its effectiveness in protecting rubber from degradation during manufacture and use, N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylene diamine (6PPD) in particular has gained ubiquity in the tire industry (Rossomme et al. 2023). It was not discovered until recently, however, that significant molar yield of 6PPD-q is produced upon ozonation of 6PPD (Hu et al. 2022). While 6PPD-q is just one of several transformation products resulting from the ozonation of 6PPD (Seiwert et al. 2022; Unice et al. 2015), it is the one that has been shown to present an environmental threat based on its toxicity to salmon, where it has been identified as the likely causative agent in urban runoff mortality syndrome (URMS) observed in the Puget Sound area of Washington state (McIntyre et al. 2021; Tian et al. 2021).

Ambient monitoring data for 6PPD and 6PPD-q in the publicly available literature remains limited. However, average concentrations of 6PPD-q in surface waters of the Seattle area were reported to be 0.09 µg/L (Zhao et al. 2023). These surface water concentrations are linked to those detected in stormwater, with a reported maximum up to 1.3 µg/L for 6PPD-q (Tian et al. 2022). Additional ambient monitoring data are needed from across the United States to fully understand the occurrence of 6PPD-q in the aquatic environment.

As more attention has been directed toward the production of the reaction product 6PPD-q via ozonation of 6PPD, knowledge of the process continues to progress beyond what has generally been described as a process of hydrolysis and photodegradation (DTSC 2022; OSPARCommission 2006; Tian et al. 2021). The very recent reinvestigation of the mechanism of 6PPD-q formation from 6PPD ozonation by Rossomme et al. (2023) provides a greater understanding of the kinetics associated with the ability of 6PPD to scavenge ozone and the specific pathway leading to production of 6PPD-q. While research is on-going as to exactly how and where the 6PPD-q formation occurs before it and other 6PPD transformation products enter water systems (DTSC 2022; Hu et al. 2022; Rossomme et al. 2023), preliminary analyses indicate that 6PPD-q is present on the exterior surfaces of tires, with the highest concentrations on the sidewalls (Patterson 2021). In support of this pathway, both 6PPD and 6PPD-q have recently been detected in samples of road dust, parking lot dust, and dust collected from inside vehicles (Huang et al. 2021), which is not unexpected given the widespread use of 6PPD as an additive in tire wear particles (TWP). The presence of TWP has been reported in road dust, soil, snow, street runoff, wastewater treatment systems, river water, and sediments, as well as in air (Baensch-Baltruschat et al. 2020).

Like its parent compound 6PPD, the formation and release of 6PPD-q from tires and TWP as tires roll across road surfaces, particularly as vehicles brake, accelerate and turn, presents a direct pathway for the release of 6PPD-q into the environment, with subsequent potential entry into aquatic systems, primarily via surface stormwater runoff (rain and snowmelt) and atmospheric deposition (Challis et al. 2021; Huang et al. 2021; Johannessen et al. 2021; Seiwert et al. 2022; Tian et al. 2021). This is now supported by numerous detections of 6PPD-q in waterways across the United States and elsewhere which clearly indicate that it is present in

aquatic systems and represents a potential risk to aquatic organisms (DTSC 2022; Tian et al. 2021). For example, 6PPD-q was detected in 57% (12/21) of stormwater samples with a mean concentration of approximately 600 ng/L and greater than 80% (28/31) of snowmelt samples with mean concentrations of 80 – 370 ng/L in Saskatoon, Saskatchewan, Canada in 2019 and 2020 (Challis et al. 2021). 6PPD-q was detected in 100% (16/16) of Seattle-region roadway runoff samples, with concentrations ranging from 800 to 19,000 ng/L (Tian et al. 2021). Noteworthy among these measurements is that 6PPD-q in receiving water samples (<300 to 3,200 ng/L) during seven storm events in three Seattle-region watersheds highly affected by URMS (Tian et al. 2021) was present at concentrations similar to receiving water samples collected from the Don River in the greater Toronto area in Southern, Ontario, Canada in the fall and winter of 2019 and 2020 (Johannessen et al. 2022). In all cases, mass loadings of 6PPD-q correlated well with roads and residential (urban) land-use area.

To date, most research on the fate and transport of 6PPD-q in the aquatic environment has centered on freshwater ecosystems, as detailed below. Similar information on the fate and transport of TWP,6PPD, and 6PPD transformation products, including 6PPD-q, in estuarine/marine environments is limited.

2.1.1 Physicochemical Properties of 6PPD-quinone

Tian et al. (2021) cited a predicted log Kow for 6PPD-q between 5 and 5.5 based on initial studies reported by Du et al. (2017), while the EPA's Estimation Program Interface (EPI) Suite software estimates a log Kow of 3.98 (DTSC 2022; U.S.EPA 2023a). Hu et al. (2023) has since determined a log Kow value for 6PPD-q of 4.3. These Kow values suggest that 6PPD-q may readily adsorb to organic matter in soils or sediments. This is consistent with findings from Spromberg et al. (2016) who found that lethal and sublethal effects of acutely toxic highway

runoff on adult coho salmon could be prevented by treatment using bioinfiltration, supporting the expectation that 6PPD-q readily binds to soil and sediment particles.

Using EPI Suite (DTSC 2022). In contrast, Hiki et al. (2021) reported that the initial concentration of 6PPD-q at its maximum water solubility was 67 ± 5 μg/L at 23°C, compared to 563 ± 204 μg/L for parent compound 6PPD. These data indicate that 6PPD-q is likely less water soluble than 6PPD, and corroborates the water solubility predicted for 6PPD-q by EPI suite. Hu et al. (2023) has since experimentally determined the water solubility of 6PPD-q to be 38 ± 10 μg/L at 20°C. Additional research is needed to further characterize the physicochemical properties and environmental fate characteristics and behavior of 6PPD-q.

2.1.2 Environmental Fate and Degradation

Hiki et al. (2021) derived a 6PPD-q half-life of 33 hours at 23°C in dechlorinated tap water. Importantly, after only four hours, the authors detected 6PPD-q in the 6PPD solution, which was attributed to the abiotic degradation of 6PPD. Consistent with previous reports (Di et al. 2022; OSPARCommission 2006), the authors also detected the 6PPD degradation product 4-hydroxydiphenylamine (4-HDPA). Tian et al. (2021) found that leachate from TWP remained toxic after it was heated to 80°C for 72 hours. Given that 6PPD-q was identified as the causal agent of the observed toxicity in their study, this suggests 6PPD-q would remain stable under similar environmental conditions. The draft EPA analytical method (1634) for the determination of 6PPD-q in aqueous matrices provides additional details regarding holding conditions for quantification of 6PPD-q in storm and surface waters (U.S.EPA 2023b).

Di et al. (2022) evaluated the hydrolysis of 6PPD-q (*rac*-6PPD-q, *S*-6PPD-q, and *R*-6PPD-q) in buffered laboratory water at 25°C, in the dark, and at different pH levels (4, 7 and 9), with observed half-lives ranging from 15.1 to 15.5 days (pH 4), 15.2 to 16.3 days (pH 7), and 14.4 to 14.7 days (pH 9). They also investigated 6PPD-q hydrolysis in river water (pH 7.7) and

found the half-life to be shorter (12.8–13.2 days) than in other buffered water solutions, which the authors speculated might be due to the different water chemistries and microbial activity. Insufficient data exist at this time to completely characterize the stability of 6PPD-q in natural waters, particularly those from different aquatic systems (lotic/lentic, freshwater/saltwater), when compared to synthetically-prepared waters. Additional research is necessary to improve our understanding of the important factors contributing to its persistence in natural surface waters. Considering data from the handful of toxicity studies in which 6PPD-q was measured throughout the exposure duration (e.g., at the start and end of the experiment), loss of 6PPD-q can be relatively high over a 24-hour exposure period. Specifically, Brinkmann et al. (2022) reported a 20 to 30% loss of 6PPD-q over 24 hours. Similarly, Lo et al. (2023) reported a loss of 18 to 54%. Lastly, Hiki and Yamamoto (2022) reported a higher percent loss of between 47 to 94%. These data indicate that more tests are needed to characterize the stability of 6PPD-q in natural waters with living organisms. Nevertheless, the limited research to date indicates the degradation of 6PPD-q in an aqueous matrix is far slower (days versus hours) than that of its parent compound, 6PPD.

The research on the potential of 6PPD-q to accumulate in aquatic life tissues is limited and challenging especially given the half-lives of 6PPD and 6PPD-q in aqueous solutions. Grasse et al. (2023) investigated the toxicokinetics of 6PPD and 6PPD-q using the zebrafish embryo model. Over 96 hours of exposure, 6PPD and 6PPD-q accumulated in the embryos with concentration factors ranging from 140 to 2,500 and 70 to 220, respectively. Semi-quantification of transformation products based on peak areas showed that within 96 hours of exposure, about 50% of 6PPD and 95% of 6PPD-q could be detoxified via biotransformation in the zebrafish embryo model (Grasse et al. 2023). Montgomery et al. (2023) hypothesized that differential

detoxification rates of various species (salmonid species and white sturgeon) may explain the highly species-specific acute lethality 6PPD-q. However, this research is very preliminary. The rapid action and quick metabolism of 6PPD-q support the use of a water screening value to protect adverse effects on aquatic life.

2.2 Measurement Endpoints

2.2.1 Overview of Toxicity Data Requirements

The *Guidelines* 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 (U.S.EPA 1985):

a. 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 four freshwater chronic toxicity studies for 6PPD-q (Appendix C and Appendix F). Therefore, a freshwater chronic screening value could not be derived at this time. However, given the short half-life of 6PPD-q 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-q literature search was conducted through the December 2023 ECOTOX update for freshwater alga or vascular plants; however, there was only one freshwater algae toxicity test for these taxa. Based on these very limited data, the EPA was unable to determine the relative toxicity of 6PPD-q to plants (see Appendix A, Appendix C, Appendix E, Appendix F, and Appendix G for freshwater species). However, the LOEC (250 µg/L 6PPD-q) observed from that study compared to the freshwater screening value (0.011 µg/L 6PPD-q) suggests plants will be protected from adverse effects (see **Section 4.3**). Therefore, this screening value was derived without the use of aquatic plant data.

2.2.2 Data Acquisition and Measure of 6PPD-q Exposure Concentrations

All acute freshwater studies with 6PPD-q-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-q 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 2022a).

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 2016a), 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-q 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-q, 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 (and given its high toxicity to specific salmonid species that are threatened or endangered in several states) 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 toxicity data landscape for 6PPD-q, the EPA has relaxed this recommendation and instead has developed the screening value using all available aquatic toxicity information meeting data quality objectives, regardless of where the species resides globally. In this context, species not resident to North America serve as taxonomically-related surrogate test organisms for the thousands of untested resident species (U.S.EPA 2022c; U.S.EPA 2022b).

2.2.2.2 Use of Nominal Concentrations

A number of 6PPD-q toxicity tests reported only nominal, or unmeasured, 6PPD-q concentrations. Given the limited availability of 6PPD-q toxicity data for aquatic life, reported nominal concentrations were used for several studies without reported measured 6PPD-q concentrations, in addition to studies reporting measured 6PPD-q 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 tests 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-q screening value, which is consistent with the *Guidelines.* Additionally, it should be noted that for 6PPD-q, all of the tests used in the screening value were measured at test initiation, all except for one test for the 7th most sensitive species, sockeye salmon (*Oncorhynchus nerka).* The test for sockeye salmon was a flow-through test, with an unbounded (greater than) toxicity value. This test was insignificant in terms of the numeric value of the LC_{50} because this value was not used quantitatively in the screening value calculation, but simply counted as part of the "N" or number of species included in the screening value calculation, since it is not in the bottom four taxa in terms of sensitivity.

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

The 6PPD-q toxicity studies in the current literature consist of a mixture of tests with concentrations measured: (1) at only 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-q at different time points throughout the experiment. At 24-hours of exposure Brinkmann et al. (2022) reported a 20 to 30% loss of 6PPD-q. Similarly, Lo et al. (2023) reported a loss after 24 hours of 18 to 54%. Lastly, Hiki and Yamamoto (2022) reported a higher percent loss after 24 hours of between 47 to 94%. These ranges were averaged for each study (i.e., average of 25% for Brinkmann et al. (2022)) and the geometric mean of the averages was 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 20% loss across studies and treatments. Therefore, for studies with nominal or initial, measured concentrations, the LC_{50} value was reduced by 20% to account for the expected loss and to make the concentrations comparable with the averaged measured concentrations. These specific adjustments are noted in the individual study summaries provided below.

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, most studies for 6PPD-q conducted tests with only 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 resulting mortality observed in definitive laboratory toxicity tests with several of the most sensitive salmonid species (e.g., Brinkmann et al. 2022; Greer et al. 2023a; Lo et al. 2023; Tian et al. 2022). Given the typical exposure durations (i.e., a few hours) associated with the onset of signs of URMS in the most acutely sensitive salmonid, coho salmon (e.g., see Chow et al. 2019; McIntyre et al. 2021; Tian et al. 2021) and the expected speed at which 6PPD-q degrades in ambient waters that are well oxygenated (see **Section 2.1**), high quality acute tests following the exposure recommendations outlined in the EPA's 850 Ecological Effects Test Guidelines were preferred; however, tests with 24 hour (or longer) exposure to 6PPD-q were considered for quantitative use.

2.2.2.5 Biomass Loading

Several studies consisted of study designs that exceeded the EPA's 850 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 (Anderson-Bain et al. 2023; Brinkmann et al. 2022; Nair et al. 2023) 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-q, 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 EPA's 850 Ecological Effects Test Guidelines), and the comparison between author-reported toxicity values and values from other acceptable tests for the same or closely-related species were considered when determining if a test without reported chemical purity could be used. The EPA reached out to the study authors to clarify the chemical purities for tests that did not report chemical purity and included the information in the individual study summaries when provided.

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 average 50% inhibition of some biological process (e.g., enzyme activity associated with an apical endpoint such as mortality) in the test organisms.

Consistent with past practice (U.S.EPA 2013), a decision rule was also applied to the 6PPD-q 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-q toxicity. Thus, the decision rule was applied as follows: "greater than" (>) high toxicity values and "less than" (<) low toxicity values were included in data calculations (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-q acute screening value for salmonids and are presented in Table 3-3 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 extremely limited. 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 6PPD-q Freshwater Aquatic Life Screening Value

During the development of this screening value for acute exposures of 6PPD-q 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 for aquatic animal species as outlined in the *Guidelines* (U.S.EPA 1985). The MDRs described in Section 2.2.1 were not met for acute freshwater criteria derivation. In addition, acceptable studies of freshwater aquatic algae and vascular plants were very limited, as well as chronic studies of freshwater animals, and acute and chronic studies of estuarine and marine animals and plants. Consequently, national 304(a) AWQC for the protection of aquatic life could not be derived for 6PPD-q at this time. However, the EPA was able derive an acute screening value for 6PPD-q 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) and that the data were aggregated at the species level, not the typical genus level averaging as recommended under the *Guidelines*, because of the extremely wide, and unusual, range of sensitivities across genus *Oncorhynchus* species. As described below, the species average LC_{50} for sensitive coho salmon (*O. kisutch*) was approximately 1,000 times lower than the least sensitive *Oncorhynchus* species average LC_{50} (Chinook salmon, *O. tshawytscha*). The *Guidelines* recommend that species values that vary by more than a factor of ten not be averaged; averaging the *Oncorhynchus* species tests would likely not be protective of the very sensitive endangered coho salmon. Thus, the EPA decided to develop the sensitivity distribution based on the individual *Oncorhynchus* species averages. The EPA chose not to develop an acute screening value based on coho sensitivity alone, as this would not reflect the full range of data available for a variety of species, with seven of the eight MDRs for various taxa met, as described below.

This assessment quantifies the toxicity of 6PPD-q to aquatic organisms to protect aquatic life in freshwater from the acute toxic effects of 6PPD-q. The 6PPD-q screening value is expected to be protective of most sensitive aquatic organisms, including sensitive salmonid

species, in the community freshwater. However, this screening value for 6PPD-q is based on more limited empirical data, including some data developed using methods not adhering to common toxicity testing guidelines (e.g., EPA's 850 Test Ecological Effects 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-q.

3 EFFECTS ANALYSIS FOR AQUATIC LIFE

All available studies relating to the acute and chronic toxicological effects of 6PPD-q on aquatic life were considered. Data for possible inclusion were obtained from published literature reporting acute and chronic exposures of 6PPD-q 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 and aquatic algae and vascular plants, as well as acute and chronic studies of estuarine and marine animals and plants, are extremely limited. Therefore, EPA was only able to derive an acute screening value for 6PPD-q in freshwater. Acute data meeting quality objectives were utilized quantitatively in deriving this screening value for acute exposures to 6PPD-q in freshwater and are presented in Appendix A; acute data considered acceptable for quantitative use in estuarine/marine exposures are presented in Appendix B. Chronic data considered acceptable for quantitative use for freshwater and estuarine/marine exposures are presented in Appendix C and Appendix D, respectively.

3.1 Summary of 6PPD-q Toxicity Studies Considered to Derive the Acute Aquatic Life Screening Value

Acute 6PPD-q toxicity data considered in deriving the acute aquatic life screening value were available for fourteen freshwater species, representing nine genera and six families (Table 3-1). The quantitatively acceptable data fulfilled four MDRs, providing data for seven salmonids (coho, sockeye, and chinook salmon, rainbow, lake and brook trout, and whitespotted char), a second family in the class Osteichthyes (zebrafish and fathead minnow), a mollusk (file ramshorn snail), and an insect (mayfly). Additionally, there were two unbounded (greater than) invertebrate study results that were qualitatively acceptable and considered adequate to fill two invertebrate MDRs (planktonic and benthic crustaceans: a pelagic cladoceran and benthic amphipod, respectively); the data on these taxa indicate they are not sensitive species, relative to

sensitive salmonid fish. Additionally, there was another unbounded (greater than) value for the white sturgeon that was qualitatively acceptable and used to fulfill one MDR (third family in the phylum Chordata). The acute value from this latter study did not rank among the most sensitive species. Because all the MDRs could not be met with quantitative data, national 304(a) AWQC for the protection of aquatic life could not be derived for 6PPD-q at this time. However, the EPA was able to derive a screening value for acute exposures of 6PPD-q in freshwater to ensure the protection of aquatic organisms, including salmon, a commercially, recreationally, and ecologically important species.

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 Freshwater and Saltwater Toxicity Datasets for 6PPD-q.

	Freshwater			
MDR[a]	**GMAV**	**SMAV**	**GMCV**	**SMCV**
Family Salmonidae in the class Osteichthyes	2	7	1	1
Second family in the class Osteichthyes, preferably a commercially or recreationally important warmwater species	2	2	0	0
Third family in the phylum Chordata (may be in the class Osteichthyes or may be an amphibian, etc.)	1[b]	1[b]	0	0
Planktonic Crustacean	1[b]	1[b]	1	1
Benthic Crustacean	1[b]	1[b]	0	0
Insect	1	1	0	0
Family in a phylum other than Arthropoda or Chordata (e.g., Rotifera, Annelida, or Mollusca)	1	1	0	0
Family in any order of insect or any phylum not already represented	0	0	0	0
Total	**9**	**14**	**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	1	1
Family in a phylum other than Chordata	1	1	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	**1**	**1**	**1**	**1**

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-q, based on the *Guidelines* approach. However, the EPA has developed a screening value for acute exposures to 6PPD-q in freshwater through use of all quantitatively- and qualitatively-acceptable acute toxicity data.

b "Greater than" (>) studies that were considered to fill MDRs from the qualitative data.

3.1.1 Summary of Acute 6PPD-q 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 Appendix A. Summaries are presented in

order of taxonomic sensitivity to 6PPD-q (Table 3-2) based on sensitivity at the genus level, with the exception of *Oncorhynchus* which is grouped by sensitivity at the species level (see specifics in Section 3.1.1.1). 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-q toxicity and/or if other MDRs could be met with those data. One qualitative fish study was acceptable for use to fulfill one of the MDRs (third family in the Phylum Chordata). Two invertebrate studies were used to fill two invertebrate MDRs (planktonic and benthic crustaceans) from the qualitative data as these studies indicate that aquatic invertebrates are not sensitive to 6PPD-q. 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-q Studies Considered for Quantitative Use.

Ranked by LC_{50} and Species and Genus Mean Acute Values (lowest to highest). Values used in SMAV/GMAV calculation are bolded.

Rank	Genus	Species	Lifestage	Method[a]	Biomass Loading (g/L)[b]	Author-Reported LC_{50} (µg/L)	EPA Adjusted / Calculated LC_{50} (µg/L)	SMAV (µg/L)	GMAV (µg/L)	Comment	Reference
1	*Oncorhynchus*	Coho salmon *Oncorhynchus kisutch*	3-week post swim up	S, M	0.337	0.041	**0.0363**	0.06134	Not Calculated[d]	Despite short duration (24 hours opposed to 96 hours)[c] the duration was considered acceptable since it mimicked realistic exposures in the aquatic environment	Lo et al. (2023)
			Juvenile, >1 yr	S, M	2.6 - 5.5	0.095	**0.07752**			Despite short duration (24 hours opposed to 96 hours)[c] the duration was considered acceptable since it mimicked realistic exposures and responses in the aquatic environment; Despite the high biomass loading[b] there was 0% mortality in controls and D.O. saturation remained > 60% during exposure; ammonia was not reported	Tian et al. (2022)
							0.09216				
			189-day old	F, M	1.3	0.0804[e]	**0.0546**			Despite short duration (24 hours opposed to 96 hours)[c] the duration was considered acceptable since it mimicked realistic exposures and responses in the aquatic environment	Greer et al. (2023a)

Rank	Genus	Species	Lifestage	Method[a]	Biomass Loading (g/L)[b]	Author-Reported LC_{50} (µg/L)	EPA Adjusted / Calculated LC_{50} (µg/L)	SMAV (µg/L)	GMAV (µg/L)	Comment	Reference
2	*Salvelinus*	Brook trout, *Salvelinus fontinalis*	Juvenile, ~1 yr	S, M	1.41	**0.59**	-	0.59	0.5590	Despite the high biomass loading[b] there was 0% mortality in controls and D.O. saturation remained > 60% during exposure. Measured ammonia was 0.13 mg/L. Despite short duration (24 hours opposed to 96 hours)[c] the duration was considered acceptable since it mimicked realistic exposures and responses in the aquatic environment	Brinkmann et al. (2022)
		Whitespotted char, *Salvelinus leucomaenis ssp. pluvius,*	Juvenile, <1 yr	R, M	4.48	0.80	**0.5709**	0.5709		Despite the high biomass loading[b] there was 0% mortality in the controls and D.O. saturation remained > 60% during exposure; ammonia was not reported.	Hiki and Yamamoto (2022)
		Lake trout, *Salvelinus namaycush*	Juvenile, 8 week post hatch	R, M	Details not provided	0.50	**0.5186**	0.5186		Despite not reporting biomass loading there was 0% mortality in the controls and D.O. saturation remained > 60% during exposure. Measured ammonia was 0.03 mg/L.	Roberts et al. 2024
3	*Oncorhynchus*	Rainbow trout, *Oncorhynchus mykiss*	Juvenile, ~2 yr	R, M	0.975	**1.00**	-	0.8087	Not Calculated[d]	Despite the high biomass loading[b] there was 0% mortality in controls and D.O. saturation remained > 60%. Measured ammonia was 0.14 mg/L.	Brinkmann et al. (2022)
			Not reported	R, M	1	2.26	**1.786**			Despite the high biomass loading[b] there was 0% mortality in controls and D.O. saturation remained > 60% during exposure; ammonia was not reported.	Di et al. (2022)

Rank	Genus	Species	Lifestage	Method[a]	Biomass Loading (g/L)[b]	Author-Reported LC_{50} (µg/L)	EPA Adjusted / Calculated LC_{50} (µg/L)	SMAV (µg/L)	GMAV (µg/L)	Comment	Reference
			Juvenile, 2 mo	R, M	0.15-0.35	0.64	**0.2961**				Nair et al. (2023)
4	*Pimephales*	Fathead minnow, *Pimephales promelas*	Adult	R, M	0.16-0.17	**>9.65**	-	>9.65	>9.65	Fed once during the exposure period	Anderson-Bain et al. (2023)
5	*Planorbella*	File ramshorn snail, *Planorbella pilsbryi*	Embryo	S, M	NA	**>11.7**	-	>11.7	>11.7	24 hour exposure with a 10 day post exposure observation period	Prosser et al. (2023)
6	*Oncorhynchus*	Sockeye salmon, *Oncorhynchus nerka*	625-day old	F, U	2.87	>50[e]	**>40.00**	>40.00	Not Calculated[d]		Greer et al. (2023a)
7	*Oncorhynchus*	Chinook salmon, *Oncorhynchus tshawytscha*	3-week post swim up	S, M	0.507	>67.31	>53.85[f]	65.68	Not Calculated[d]		Lo et al. (2023)
			582-day old	F, M	1.91	82.1[e]	**65.68**				Greer et al. (2023a)
8	*Danio*	Zebrafish, *Danio rerio*	Embryo	R, U	NA	132.9	**106.3**	106.3	106.3	Zebrafish embryo test biomass loading was not an issue	Varshney et al. (2022)
9	*Hexagenia*	Mayfly, *Hexagenia sp.*	Larva	S, M	NA	**>232.0**	-	>232.0	>232.0	Mixture of two species	Prosser 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 (U.S.EPA 2016b).

[c] 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 (U.S.EPA 2016b).

[d] GMAV for *Oncorhynchus* was not calculated given the disparate SMAVs in this genus per the *Guidelines*.

[e] Values were calculated from sequential replication.

[f] Not included in SMAV calculation because a definitive value was available.

3.1.1.1 Most acutely sensitive taxon: *Oncorhynchus kisutch* (Coho Salmon – salmonid fish)

Lo et al. (2023) conducted a 24-hour static, measured acute test of 6PPD-q (97.26% purity) with the coho salmon, *Oncorhynchus kisutch*. Juvenile coho salmon (3 weeks post-swim up; average body weight 0.43 g wet weight (ww)) were used for testing. The 6PPD-q exposures consisted of five nominal concentrations of 11.9, 21.4, 38.6, 69.4, 125 ng 6PPD-q/L) and separate well water and solvent (0.01% ethanol) controls, with four replicates each. Test concentrations of 6PPD-q were measured in two replicates from each of the treatment groups at the initiation and termination of the test (the same replicates at each sampling event). Average concentrations of 6PPD-q at test initiation deviated 22.8 ± 16.9% (3.3 – 49.3%) from the nominal concentration; average loss of 6PPD-q reported after 24 hours of exposure was 35.2 ± 17.1%. Survival in the controls was 100% across all four replicates. Survival in one replicate of the solvent controls was 71.4% as fish exhibited symptoms consistent with exposure to 6PPD-q, and with authors reporting 6PPD-q contamination (34.4 ng/L) in that tank; data from the contaminated replicate was excluded from analyses. The remaining three solvent control replicates exhibited 100% survival. The authors noted that some 6PPD-q exposed fish exhibited symptoms (e.g., gasping, loss of equilibrium, erratic swimming) and mortality during the fourth hour of exposure at the highest test concentration (104.7 ng/L) with 100% mortality in two of the four replicates; overall average survival in the highest test concentration was 7.1% across all four replicates. The author-reported 24-hour LC_{50} was 0.041 µg 6PPD-q/L, which was based on initial measured concentrations, Study authors performed a degradation study due to observed holding time loss of 6PPD-q. The results from the degradation study were natural-log transformed and fitted to a linear regression to determine a slope by which the measured toxicity concentration values at the time of initiation could be estimated from for the author-reported

LC_{50} value reported above. The EPA curve fit the concentration-response (C-R) data to calculate a LC_{50} value based on average concentrations instead of initial concentrations. The EPA-calculated LC_{50} was 0.0363 µg/L 6PPD-q, which was acceptable for quantitative use.

Tian et al. (2022) evaluated the toxicity of 6PPD-q (98.8% purity) to juvenile coho salmon (*O. kisutch*) for 24 hours under static measured conditions. Juvenile coho salmon (>1 year old, 30-64 g) were tested in 70 L aquaria with six fish per aquarium. The biomass loading rate of 2.6-5.5 g/L exceeds that recommended in the EPA's 850 Ecological Effects Test Guidelines (0.8 g/L). While the biomass loading rate was over 3.2 times higher than the 0.8 g/L rate recommended in the EPA's 850 Ecological Effects Test Guidelines (U.S.EPA 2016a), there were no signs of stress in the test (solvent) control organisms (0% mortality) and D.O. saturation remained greater than 60% during the exposure in all test aquaria. The LC_{50} was calculated by combining the results of four separate exposure series using different combinations of six concentrations of 6PPD-q and a negative control. In the first series treatments were more widely spaced, while the remaining three series were more narrowly focused within the concentration range where partial mortality was expected. Exposures were serially-repeated in triplicate across three weeks. Nominal and measured exposure concentrations for each series were: Batch 0: 20, 35.6, 63.2, 112.5, 200 and 23.1, 41.1, 73.0, 130, 231 ng/L 6PPD-q, respectively; Batch 1: 50, 66, 87, 115, 152, 200 and 53.2, 68.9, 97.1, 122, 162, 226 ng/L 6PPD-q, respectively; Batch 2: 60, 72, 86, 104, 125, 150 and 68.1, 83.5, 99.3, 115, 154, 194 ng/L 6PPD-q, respectively; and Batch 3: 60, 72, 86, 104, 125, 150 and 70.5, 84.6, 105, 124, 151, 190 ng/L 6PPD-q, respectively. The authors note that the measured concentrations were systematically higher than the nominal concentrations (median 16%), potentially caused by the volumetric vessels (Hamilton syringes and glass pipettes). No mortality was observed in the solvent control in any exposure series. The

author-reported 24-hr LC_{50} was 0.095 µg 6PPD-q/L and was based on initial measured concentrations only and combining each experimental series into one C-R curve. The EPA curve fit the data based on each experimental series (batch reported in the paper). The LC_{50} values from each series that could be curve fit were used as opposed to combining all series into one curve fit. The EPA-calculated LC_{50}s were adjusted to lower the value by 20% in order to account for loss of 6PPD-q over the 24 hour test duration. The adjusted EPA-calculated LC_{50}s were 0.07752 and 0.09216 µg/L 6PPD-q, which were acceptable for quantitative use.

Greer et al. (2023a) conducted a 24-hour partially-measured, flow-through acute test of 6PPD-q (97.5% purity) with the coho salmon, *O. kisutch*. Young-of-year coho salmon (189 days old; average initial body weight of 1.95 g ww) were exposed to 6PPD-q in 9 L aerated tanks containing 8 L of with water temperature maintained at 8°C. 6PPD-q concentrations ranging from 25 to 125 ng/L (n = 20 exposures) over the course of multiple days, with 2-3 tanks tested per day, each with a different 6PPD-q concentration. Replication was done serially, across different days. Both nominal and measured concentrations were used to fit the curve for the coho LC_{50}. There were five measured and 15 nominal exposures evaluated in total; however there were multiple nominal concentrations tested at different times and nominal-measured pairings not always the same. Specific concentration pairings are provided in the paper. No mortality was observed in any solvent control treatments. The author-reported 24-hour LC_{50} for young-of-year coho salmon was 80.4 ng 6PPD-q/L, or 0.0804 µg/L. The author-reported value was a combination of measured and nominal concentrations. The EPA curve fit the data to calculate an LC_{50} value based on measured average concentrations only. The EPA-calculated LC_{50} was 0.0546 µg/L 6PPD-q, which was acceptable for quantitative use.

3.1.1.1.1 Oncorhynchus kisutch SMAV calculation

The geometric mean of the four values from the three studies was calculated for the coho salmon SMAV. The SMAV was calculated based on the following EPA-calculated LC_{50} values for *O. kisutch*: 0.0363 µg/L from Lo et al. (2023), 0.0546 µg/L from Greer et al. (2023a), and 0.07752 and 0.09216 µg/L from Tian et al. (2022), respectively. The resulting SMAV of 0.06134 µg 6PPD-q/L (61.34 ng/L) represents the most sensitive mean acute value in the EPA's freshwater acute dataset for 6PPD-q. The SMAV for *Oncorhynchus mykiss* is presented separately below (see Section 3.1.1.3) and was also used in the derivation of the screening value.

3.1.1.2 Second most acutely sensitive genus: *Salvelinus* (Salmonid)

Brinkmann et al. (2022) investigated the acute toxicity of 6PPD-q (97% purity) to brook trout, *Salvelinus fontinalis* in a static, measured exposure. Juvenile brook trout (~1 year old, 17.1 cm, 52.8 g) were statically exposed to solvent controls and five measured concentrations of 6PPD-q (0.11, 0.72, 1.35, 2.21, and 4.35 µg/L) for 24 hours in 150 L inert glass fiber Krescel tanks (two replicate tanks with four fish each for 6PPD-q treatments and four replicates with four fish each for solvent controls; 56 fish total). The biomass loading rate was 1.41 g/L, which is almost two times higher than the EPA's 850 Ecological Effects Test Guidelines recommended rate of 0.8 g/L (U.S.EPA 2016b). Nominal and average measured exposure concentrations were 0.1, 0.5, 1, 2 and 4, and 0.11, 0.72, 1.35, 2.21 and 4.35 µg/L 6PPD-q, respectively. No control mortality was observed. There was 100% mortality in the high treatment group within three hours of exposure. The author-reported 24-hour LC_{50} was 0.59 µg 6PPD-q/L. The EPA was unable to curve fit the data based on the level of detail provided in the paper and there is no change in the value since the author-reported value was based on average concentrations over the experimental duration. Consistent with the study review approach described in Section 2.2.2 above, this test was classified as quantitative despite the short duration (24 hours as opposed to

96 hours), and the high biomass loading rate (since the test organisms did not appear to be stressed from the loading rate used and D.O. measurements were at acceptable levels).

One of three species evaluated by **Hiki and Yamamoto (2022)** for 6PPD-q toxicity was the whitespotted char, *Salvelinus leucomaenis ssp. pluvius*, via a static-renewal, measured exposure. This species is not a resident species in North America, but other species in this genus are resident species. 6PPD-q (>95% purity, Cambridge Isotope Laboratories) was dissolved in acetonitrile prior to testing. Acute static-renewal measured lethality tests were performed according to the OECD Test Guideline 203 with slight modifications noted below that were inconsequential to the decision to use the study result in the derivation of this screening value. Seven juvenile fish <1 year old (average of 3.2 g/fish, average length of 7.0 cm) were exposed for 96 hours in a glass tank containing approximately 5 L of dechlorinated tap water spiked with five concentrations (0.16, 0.36, 0.82, 1.3, and 3.5 µg/L) of 6PPD-q measured at the start of the experiment. There was one exposure chamber for each treatment group. Although two or more replicate exposure chambers per treatment are preferred, one chamber is acceptable within the EPA's 850 Ecological Effects Test Guidelines. The biomass loading rate was 4.48 g/L, which is over five times higher than the EPA's 850 Ecological Effects Test Guidelines recommended rate of 0.8 g/L (U.S.EPA 2016b). The carrier solvent control was dosed with acetonitrile at 0.012% (v/v). Every 24 hours surviving fish were transferred to a glass tank containing 5 L of a newly prepared test solution to keep exposure concentrations stable. The measured 6PPD-q concentrations were 54-109% of the nominal concentrations just after water renewal. Concentrations of 6PPD-q were also measured at the start and end of each test solution renewal period, and it was determined that 47-97% of the detected 6PPD-q was lost within the first 24 hours of water renewal. All observed lethality occurred within 24 hours of exposure at all

concentrations and there was no solvent control mortality. The author-reported 96-hour LC_{50} was 0.80 µg 6PPD-q/L, based on the measured concentrations at the start of the water renewal; the author-reported 96-hour LC_{50} was 0.51 µg /L 6PPD-q, based on time weighted average concentrations. The EPA curve fit the C-R data to calculate an LC_{50} value based on the time weighted average concentrations. There were differences in the model fits between the EPA and the author-reported LC_{50}. This study was deemed acceptable for quantitative use despite the high biomass loading as there was 0% mortality in the controls and D.O. saturation remained > 60% during the exposure in all test solutions, indicating that the test organisms were not stressed by this biomass loading rate. The EPA-calculated LC_{50} was 0.5709 µg/L 6PPD-q, which was acceptable for quantitative use.

Roberts et al. (2024) examined the effects of 6PPD-q (97% purity) on lake trout (*Salvelinus namaycush*) in a 96-hour acute static-renewal, measured exposure. Each test chamber (2.5 L tanks of unspecified material) received one of four nominal concentrations of 6PPD-q (0.1, 0.3, 0.9, and 2.7 µg/L) or a control, for a total of 25 tanks. All test concentrations and controls included 0.01% dimethyl sulfoxide (DMSO) as a solvent. Exposure water was sampled in each tank at the beginning of the experiment, after 24 hours, and three additional times during the experiment. Time weighted average 6PPD-q concentrations during the 96-hour exposure period were <0.10, 0.16, 0.55, and 2.1 µg/L. A concentration of 0.05 µg/L, or half of the detection limit, was used to represent the lowest treatment level when calculating the acute LC_{50}. Fifteen eight-week-old post-hatch juvenile lake trout were randomly added to each tank. Acute mortality was observed at the highest test concentration within the first hour and continued throughout the test. Behavioral changes were observed at the 0.55 and 2.1 µg/L test concentrations throughout the study, including loss of coordination, gasping, and surface

swimming. All fish that exhibited these behaviors died within six hours. No fish in the control or two lowest treatment concentrations exhibited any behavioral changes, and control survival was 100%. The author calculated LC_{50} was 0.50 µg/L (based on time weighted average concentrations). The EPA re-calculated the LC_{50} from C-R data based on time weighted average concentrations. The EPA-calculated LC_{50} was 0.5186 µg/L 6PPD-q, which was acceptable for quantitative use.

3.1.1.2.1 Salvelinus GMAV calculation

The author-reported LC_{50} of 0.59 µg 6PPD-q/L for *Salvelinus fontinalis,* the EPA-calculated LC_{50} of 0.5709 µg 6PPD-q/L for *Salvelinus leucomaenis ssp.pluvius*, and the EPA-calculated LC_{50} of 0.5186 µg 6PPD-q/L for *Salvelinus namaycush* were used as SMAVs, since there were no other quantitative toxicity values available for these species. The geometric mean of these three LC_{50} values were used to calculate a GMAV of 0.5590 µg 6PPD-q/L for *Salvelinus,* which represents the second most sensitive mean acute value in the EPA's freshwater acute dataset for 6PPD-q.

3.1.1.3 Third most acutely sensitive taxon: *Oncorhynchus mykiss* (rainbow trout – salmonid fish)

Brinkmann et al. (2022) performed a 96-hour static-renewal, measured acute test of 6PPD-q (97% purity) with the rainbow trout, *Oncorhynchus mykiss*. The test chambers were 700 L glass-fiber Min-o-Cool tanks with 500 L of test solution per tank. Each tank contained five fish (~2-year-old juveniles, average of 97.5 g), with two replicate tanks per treatment and a control that was replicated three times. The biomass loading rate averaged 0.975 g/L, slightly higher than the 0.8 g/L recommended in the EPA's 850 Ecological Effects Test Guidelines (U.S.EPA 2016b). Stock solution was added to tanks to make five treatment concentrations (0.09, 0.72, 1.38, 2.78 and 5.33 µg/L average measured 6PPD-q) plus a DMSO solvent control. Average

measured test concentrations were based on the quantification of 6PPD-q in samples of test solution collected before and after each renewal. With the exception of the lowest test concentration, measured concentrations were within ± 12% of nominal. The lowest concentration was within 27% of the nominal concentration. Measured concentrations decreased between daily solution renewals by an average 44.2% at the lowest concentration and 26.5% and 26% at the two next higher concentrations. Nominal and average measured exposure concentrations were 0.15, 0.75, 1.5, 3 and 6, and 0.09, 0.72, 1.38, 2.78 and 5.33 µg/L 6PPD-q, respectively. No solvent control mortality was observed. The author-reported 96-hour LC_{50} was 1.00 µg 6PPD-q/L based on average concentrations over the experimental duration. The EPA was unable to curve fit the data based on the level of detail provided in the paper, and therefore, the author-reported LC_{50} was used. This test was determined to be acceptable for quantitative use despite the slightly elevated biomass loading rate compared to the EPA's 850 Ecological Effects Test Guidelines since there was no control mortality and D.O. saturation was 92.8%.

Di et al. (2022) performed a 96-hour static-renewal, measured acute test of 6PPD-q (≥98% purity) with the rainbow trout, *O. mykiss*. Stock solution was added to test chambers containing 24 L of dechlorinated water to make five treatment concentrations (1.75, 1.92, 2.12, 2.33, and 2.56 µg/L measured 6PPD-q) plus an acetonitrile solvent control. There were three tanks per treatment (and control). Each tank contained eight fish (juveniles, 3.0±0.3 g) resulting in an average biomass loading rate of 1 g/L, slightly higher than that recommended in the EPA's Ecological Effects Test Guidelines of 0.8 g/L (U.S.EPA 2016b). A 50% water exchange was conducted after 48 hours. The study authors did not report a comparison of measured concentrations to nominal. The author-reported 96-hour LC_{50} was 2.26 µg/L 6PPD-q. The EPA re-calculated the LC_{50} (2.232 µg/L) from C-R data based on measured concentrations provided

by study authors in the paper, but it is unclear when these measurements were taken during the exposure. It was assumed that reported measured concentrations were initial concentrations; therefore, the EPA-calculated LC_{50} value was adjusted to lower the value by 20% in order to account for loss of 6PPD-q over the experiment. The adjusted EPA-calculated LC_{50} was 1.786 µg/L 6PPD-q. This study was determined to be acceptable for quantitative use despite the slightly elevated biomass loading rate since the test organisms did not appear to be stressed (0% control mortality, D.O. saturation > 60%).

Nair et al. (2023) conducted a 96-hour measured, static acute test of 6PPD-q (95% purity) with the rainbow trout, *Oncorhynchus mykiss*. The acute toxicity test was conducted using juvenile rainbow trout (2 months old, 0.3- 0.7 g). Rainbow trout were exposed to five nominal concentrations (0.2, 0.8, 3, 12 and 25 µg/L) of 6PPD-q with measured concentrations of less than detection limit, 0.29, 1.94, 11.2 and 40.0 µg/L 6PPD-q, respectively. There were 10 fish in each replicate. All fish exposed to the nominal concentration of 0.8 µg/L survived the first day of exposure, but mortality increased from 30% at Day 2, to 43% at Day 4. The author-reported 96-hour LC_{50} was 0.64 µg 6PPD-q/L. The EPA-calculated LC_{50} (0.3701 µg/L) was based on measured concentrations provided by study authors in the paper, but it was unclear when these measurements were taken during the exposure. The reported measured concentrations were assumed to be based on initial measured concentrations; therefore, the EPA-calculated LC_{50} value was adjusted to lower the value by 20% in order to account for loss of 6PPD-q over the experiment duration. The adjusted EPA-calculated LC_{50} was 0.2961 µg 6PPD-q/L, which was acceptable for quantitative use.

3.1.1.3.1 Oncorhynchus mykiss SMAV calculation

The geometric mean of the author-reported LC_{50} and the two adjusted EPA-calculated LC_{50} values for *O. mykiss* (1.00 µg/L, 1.786 µg/L and 0.2961 µg/L, respectively) were used to

calculate an SMAV of 0.8087 µg/L, which represents the third most sensitive mean acute value in the EPA's freshwater acute dataset for 6PPD-q. As mentioned, in Section 3.1.1.1.1, this SMAV was not combined with the other tested species of this genus to calculate a GMAV, as the *Guidelines* state that SMAVs should not differ by more than a factor of 10 for the calculation of GMAVs. As such, the four SMAVs for *Oncorhynchus* (which differ by a factor >1,000) were treated as separate values representing the different sensitivities of the four tested *Oncorhynchus* species. Therefore, the SMAV of 0.8087 µg 6PPD-q/L for *O. mykiss* was used in the derivation of the screening value instead of an *Oncorhynchus* GMAV.

3.1.1.4 Fourth most acutely sensitive genus: *Pimephales* (minnow)

Anderson-Bain et al. (2023) performed a 96-hour static-renewal, measured acute test of 6PPD-q (97% purity) with fathead minnow, *Pimephales promelas*. Following acclimation, adult fathead minnows were exposed for 96 hours in duplicate (2 tanks per treatment, 6 fish per tank) to 6PPD-q at nominal concentrations of 0, 0.2, 2, or 20 µg/L. A 50% water renewal was conducted daily to replenish concentrations of 6PPD-q. A 990 µL sample of water was taken before and one hour after addition of 6PPD-q, and immediately before and after each water change. The time-weighted average concentrations of 6PPD-q measured over the 96-hour exposure were 0.09, 0.85, and 9.65 µg/L, which were approximately 44, 43 and 48 percent of nominal values in the low, medium, and high treatment groups, respectively. The final concentration of dimethyl sulfoxide (DMSO) in all tanks was 0.01% (v/v). Exposures included both water only and solvent controls (0.05% DMSO). Exposure to 6PPD-q for 96 hours did not cause mortality of adult fathead minnows. The author-reported 96-hour LC_{50} was >9.65 µg 6PPD-q/L, based on average concentrations over the experimental duration used. The EPA was unable to curve fit the data based on the level of detail and lack of effects provided in the paper. The author-reported LC_{50} was acceptable for quantitative use.

3.1.1.4.1 Pimephales GMAV calculation

As no other quantitative toxicity values were available for this species or genus. The author-reported LC_{50} of >9.65 µg 6PPD-q/L served directly as the mean acute value (SMAV and GMAV).

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

There is only one quantitatively acceptable test for acute 6PPD-q toxicity with an estuarine/marine species.

3.1.2.1 Most acutely sensitive genus: *Parhyale* (amphipod)

Botelho et al. (2023) performed a 96-hour static, unmeasured acute test of 6PPD-q (≥97% purity) with the amphipod, *Parhyale hawaiensis*. Neonates (≤ 7 days old) used for testing were obtained from in-house cultures. 6PPD-q was evaluated in five concentrations (31.25 to 500 µg/L). A stock solution of 6PPD-q was prepared in dimethyl sulfoxide (DMSO) at the solubility limit (5 g/L). Dilutions in the test media contained a maximum concentration of 0.01% DMSO as recommended by OECD (2019). Artificial seawater was used as negative control, and 0.01% DMSO as the solvent control. The test was conducted in 96-well plates and for each concentration, 32 neonates were exposed for 96 hours (1 neonate per well). Control organism mortality was ≤3.1% in the artificial seawater and 0% in the solvent control. Across all 6PPD-q treatments mortality was ≤3.1%. The 96-hour LC_{50} was >500 µg 6PPD-q/L, which was acceptable for quantitative use.

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

There are two quantitatively acceptable empirical tests for chronic 6PPD-q toxicity with freshwater animal species.

3.1.3.1 Most chronically sensitive genus: *Salvelinus* (trout)

Roberts et al. (2024) examined the effects of 6PPD-q (97% purity) on lake trout (*Salvelinus namaycush*) in a 45-day static-renewal measured chronic exposure. Test solution was added to 2.5 L tanks (test chambers), which were individually aerated and allowed to equilibrate for 24-hours prior to test initiation. Each tank received one of six nominal concentrations of 6PPD-q (0.625, 1.25, 2.5, 5, 10, and 20 µg/L) or a control, for a total of 35 tanks. All test concentrations and controls included 0.01% DMSO as a solvent. Exposure water was sampled in each tank 24 hours before the experiment, at the beginning of the experiment, and four separate times during the experiment. Time weighted average 6PPD-q concentrations during the 45-day exposure period were (0.22, 0.58, 1.3, 3.4, and 13.5 µg/L). No 6PPD-q was reported in the solvent controls. Fifteen newly-hatched lake trout alevins were randomly added to each tank. Relatively high mortality was observed during the first four days of the exposure and continued throughout the test. Behavioral changes were not observed; however, deformities were. Deformities occurred as early as three days after testing began, suggesting that 6PPD-q may disrupt growth and development during early life stages. The most common deformity was yolk sac edema, followed by blood pooling and spinal curvature. The highest incidences of yolk sac edema and blood pooling in the caudal fin and eye were at the 3.4 µg/L treatment level, and the highest incidences of spinal curvature were at the 1.3 and 7.6 µg/L treatment level. The authors reported that there were no statistically significant differences in fish length or weight among treatments for surviving fish. Control survival was greater than 80%. The author-reported chronic endpoint was 45-day survival (LC_{50}). The author-calculated LC_{50} of 0.39 µg/L (based on time weighted averages) 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: *Daphnia* (cladoceran)

Prosser et al. (2023) performed a 21-day static-renewal, measured chronic test of 6PPD-q (>99.8% purity) with the cladoceran, *Daphnia magna*. Organisms used in this study were obtained from a continuous culture that was maintained at the University of Guelph. The chronic toxicity test with *D. magna* was adapted from the Organization of Economic Cooperation and Development (OECD) standardized test method 211 (OECD 2012). To simplify the test, only mortality and growth were assessed, the number of neonates produced was not assessed in this test. Exposure solutions were prepared from a concentrated stock solution of 6PPD-q. The culture water containing *C. vulgaris* and *R. subcapitata* was spiked with the stock solution of 6PPD-q (>99.8% purity) to create the different exposure solutions used in the test. A negative control, solvent control (0.02% methanol by volume) and 10, 50, and 100 µg/L treatments were prepared. One neonate (<24 hours old) was placed in each test vessel (50 mL glass test tube) and ten replicate test vessels were prepared for each treatment. Every two days, test organisms were transferred with a glass Pasteur pipette into test vessels with new exposures solutions. The test was terminated after 21 days, and the size of surviving organisms was measured. At the end of the 21-day exposure, the size of daphnids were measured to assess growth. When new exposure solutions were made on day 0, 8, and 21, they were sampled and analyzed for 6PPD-q before conducting the solution change. The measured concentration of 6PPD-q [quantified using solid phase extraction followed by ultraperformance liquid chromatography (UPLC) and tandem mass spectrometry (MS-MS)] was consistently lower than the nominal concentration. The percentage difference between measured and nominal concentration at day 0 ranged from 58.0% to 71.0%. Control organism survival was 100% and was ≥90% for all 6PPD-q treatments. The 21-day NOEC for mortality and growth/length was 30.2 µg 6PPD-q/L (based on the average measured concentrations for the highest test concentration), which was acceptable for quantitative

consideration in development of a screening value(had other taxa data been sufficient to develop a chronic screening value). Based on the sampling design (limited number of test concentration measurements) this effect concentration is likely lower due to 6PPD-q loss in the test system.

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

There is one quantitatively acceptable empirical test for chronic 6PPD-q toxicity with an estuarine/marine species.

3.1.4.1 Most chronically sensitive genus: *Brachionus* (rotifer)

Maji et al. (2023) conducted a 7-day unmeasured, static-renewal chronic test of 6PPD-q (purity not reported) with the marine rotifer, *Brachionus koreanus*. A stock solution was prepared in acetone followed by successive dilutions in artificial seawater at 30 psu. Since acute toxicity was not observed in *B. koreanus* in response to 6PPD-q, chronic toxicity was assessed based on life-cycle parameters such as population growth and fecundity of the rotifer. For chronic experiments, neonate rotifers hatched within 2 hours were used. Individual neonate rotifers (body size <120 µm) were placed in each well of a six-well culture plate containing 50, 250, 500 or 1,000 µg/L of 6PPD-q. Stock solutions were made in acetone; details about the use of a solvent or water only controls were not provided. To determine the population growth, the number of rotifers in each well was counted under a stereomicroscope (SZX7, Olympus, Tokyo, Japan) at 24-hour intervals until no further population growth was observed. For fecundity measurements, newborn rotifers were counted using the SZX7 stereomicroscope every 12 hours as an indicator of reproduction until the mature rotifer died. All experiments were conducted in triplicate and test solutions were renewed daily. The 7-day population growth rate NOEC was 1,000 µg 6PPD-q/L, which was acceptable for quantitative consideration in a screening value

development if the chemical purity of 6PPD-q is identified to be greater than 80% via communication with the study authors.

3.2 Derivation of Aquatic Life Screening Values for 6PPD-q

3.2.1 Derivation of Acute Screening Value for Freshwater

There are insufficient data to derive a national recommended freshwater AWQC for 6PPD-q. There are currently nine quantitatively acceptable acute values for freshwater taxa (five GMAVs and four *Oncorhynchus* sp. SMAVs). *Oncorhynchus* is represented in the sensitivity distribution at the species level. The EPA's *Guidelines* recommends acceptable toxicity values for at least eight families of aquatic animals be available to fulfill the eight MDRs in order to calculate a criterion value. The quantitative acute values only fulfill four of the eight MDRs. Empirical data are currently very limited for 6PPD-q and 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 those of the ASTM or OECD. For example, most testing on fish was conducted with 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-q. 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-q to aquatic organisms, the EPA developed an acute protective screening value for 6PPD-q 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-q which has been found to be extremely toxic to certain sensitive aquatic species, including sensitive salmonid fish. This screening value was calculated generally following the traditional (*Guidelines*) approach with limited adaptations, which are summarized in Section 2.2.2. This

includes the aggregation of toxicity data at the species level for *Oncorhynchus* species, instead of the typical genus-level data aggregation conducted under the *Guidelines* methods. This species-level data aggregation was used because the toxicity 6PPD-q ranged very widely, approximately 1,000-fold, across the sensitive *Oncorhynchus* species (salmon); thus, averaging at the genus level would not have been protective of the more sensitive species in the genus. This separation of the *Oncorhynchus* species is consistent with direction in the *Guidelines* to avoid averaging widely disparate data (more than 10-fold different values). This screening value was derived by calculating the freshwater final acute value (FAV), the 5th percentile of the species/genus sensitivity distribution calculated using the general approach described in the *Guidelines* (U.S.EPA 1985) (see Table 3-3, Figure 3-1). Specifically, the FAV was calculated using the SMAV of 0.06134 µg/L for *Oncorhynchus kisutch*, the GMAV of 0.5590 µg/L for *Salvelinus*, the SMAV of 0.8087 µg/L for *Oncorhynchus mykiss* and the GMAV of >9.65 µg/L for *Pimephales*, with the total number of unique taxa SMAVs and GMAVs (inclusive of three qualitative mean acute values, refer to Section 3.1) equal to 12 (e.g., n=12). The FAV for 6PPD-q was determined to be 0.02138 µg 6PPD-q/L, which is lower than all of the SMAVs/GMAVs of the tested species (Table 3-4). The FAV was then divided by two to obtain a concentration yielding minimal effects (see Section 2.3.1). The FAV/2, which is the screening value for acute exposures of 6PPD-q in freshwater, was determined to be 11 ng/L (or 0.011 µg/L). This 11 ng/L (parts per trillion) value is expected to be protective of freshwater genera exposed to 6PPD-q 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 Species/Genus Mean Acute Values.

Rank[a]	GMAV[b] (µg/L)	MDR Group[c]	Genus	Species	SMAV (µg/L)
1	Not calculated (SMAVs used)	A	*Oncorhynchus*	Coho salmon, *Oncorhynchus kisutch*	0.06134
2	0.5590	A	*Salvelinus*	Brook trout, *Salvelinus fontinalis*	0.59
				Whitespotted char, *Salvelinus leucomaenis ssp. pluvius*	0.5709
				Lake trout, *Salvelinus namaycush*	0.5186
3	Not calculated (SMAVs used)	A	*Oncorhynchus*	Rainbow trout, *Oncorhynchus mykiss*	0.8087
4	>9.65	B	*Pimephales*	Fathead minnow, *Pimephales promelas*	>9.65
5	>11.7	G	*Planorbella*	File ramshorn snail, *Planorbella pilsbryi*	>11.7
6	>12.7[d]	C	*Acipenser*	White sturgeon, *Acipenser transmontanus*	>12.7
7	Not calculated (SMAVs used)	A	*Oncorhynchus*	Sockeye salmon, *Oncorhynchus nerka*	>40
8	>43[d]	E	*Hyalella*	Amphipod, *Hyalella azteca*	>43
9	>46[d]	D	*Daphnia*	Cladoceran, *Daphnia magna*	>46
10	Not calculated (SMAVs used)	A	*Oncorhynchus*	Chinook salmon, *Oncorhynchus tshawytscha*	65.68
11	106.3	B	*Danio*	Zebrafish, *Danio rerio*	106.3
12	>232.0	F	*Hexagenia*	Mayfly, *Hexagenia sp.*	>232.0

a Ranked from the most sensitive to the most resistant based on Mean Acute Value (SMAV and GMAV).
b From Appendix A: Acceptable Freshwater Acute 6PPD-q Toxicity Studies.
c MDR Groups – Freshwater:

A. a 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 maybe 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.

d Greater than values that were considered to fill MDRs from the qualitative data. Study summaries included in Section 4.2.1.

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

Calculated Freshwater FAV based on 4 lowest values: Total Number of unique taxa SMAVs and GMAVs in Data Set = 12						
Rank	**Genus or Species**	**SMAV or GMAV (µg/L)**	**ln(GMAV)**	**ln(GMAV)^2**	**P=R/(N+1)**	**sqrt(P)**
1	*Oncorhynchus kisutch*	0.06134	-2.7913	7.7915	0.0769	0.2774
2	*Salvelinus*	0.5590	-0.582	0.34	0.1538	0.3922
3	*Oncorhynchus mykiss*	0.8087	-0.212	0.05	0.2308	0.4804
4	*Pimephales*	9.65	2.267	5.14	0.3077	0.5547
		Σ (Sum):	**-1.32**	**13.31**	**0.769**	**1.705**

S^2 =	301.21	S = slope	
L =	-7.726	L = X-axis intercept	
A =	-3.845	A = lnFAV	
FAV =	0.02138	P = cumulative probability	
(FAV/2) =	**0.011 µg/L 6PPD-q; 11 ng/L** (rounded to two significant figures)		

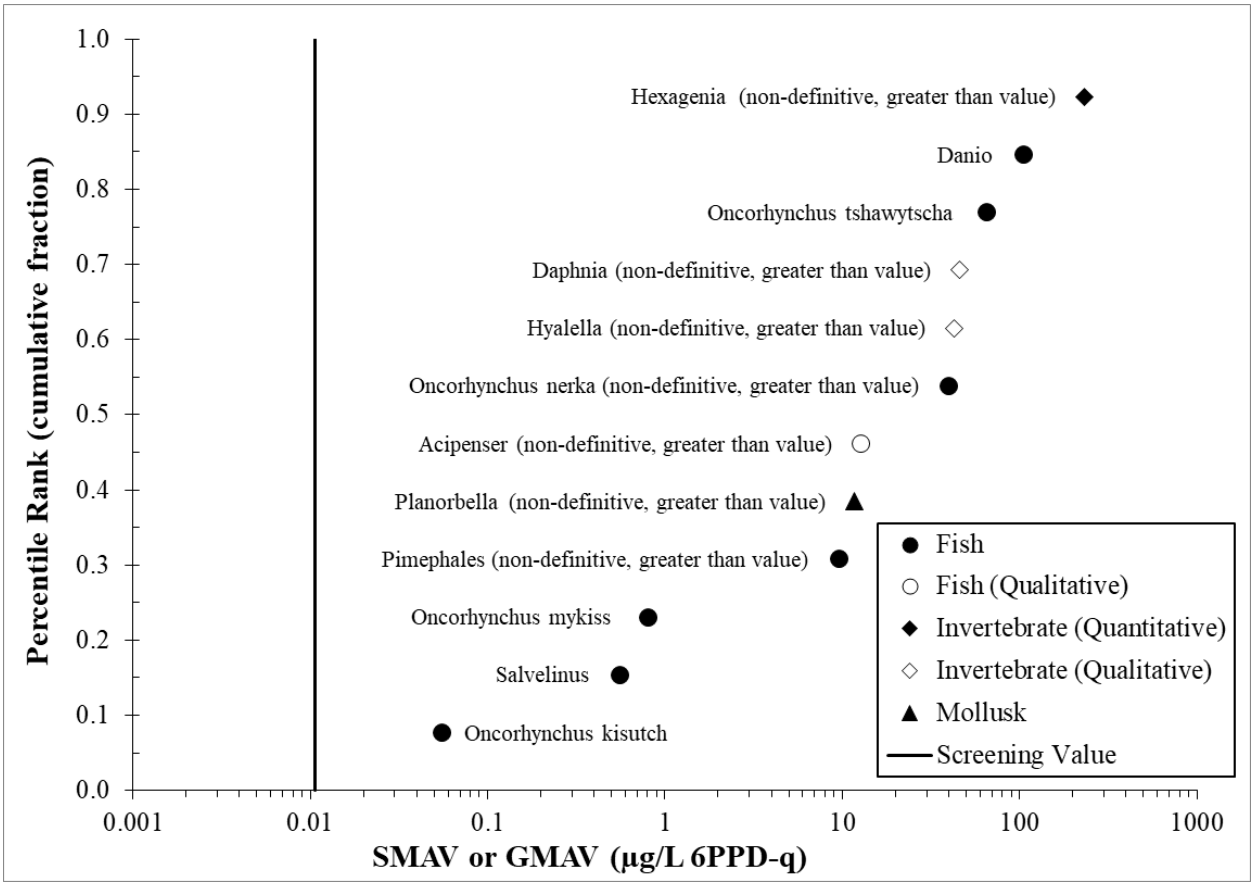

Figure 3-1. Ranked Freshwater 6PPD-q Mean Acute Values Fulfilling the Eight Family MDRs Used to Derive Screening Value for Acute Exposures.
The studies associated with the four most sensitive mean acute values were summarized above in Section 3.1.1 and the qualitative studies are summarized below in Section 4.2.

3.2.2 Derivation of Acute Water Screening Value for Estuarine/Marine Water

Not enough data exist to calculate an estuarine/marine FAV.

3.2.3 Derivation of Chronic Screening Value for Freshwater

Not enough data exist to calculate a chronic freshwater FCV.

3.2.4 Derivation of Chronic Screening Value for Estuarine/Marine Water

Not enough data exist to calculate a chronic estuarine/marine FCV.

3.3 Summary of Acute 6PPD-q Freshwater Aquatic Life Screening Value

The aquatic life screening value for 6PPD-q derived in this document includes a water-column based acute screening value for freshwaters. A chronic screening value for freshwaters

and acute and chronic 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-q 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-q in freshwater is 11 ng/L (0.011 µg/L) (Table 3-5). As part of deriving the screening value for 6PPD-q, 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-q 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-q for short durations (e.g., one hour or less). 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-q Aquatic Life Screening Value.

Type/Media	Acute Water Column Screening Value[1]
Magnitude	11 ng/L (0.011 µg/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 Analysis Supporting the Derivation of the Screening Value for Acute Exposures of 6PPD-q in Freshwater

In addition to the EPA's screening value for acute exposures of 6PPD-q in freshwater of 11 ng/L (0.011 µg/L) as described above in Section 3.3, an additional analysis was completed to evaluate the effect of calculating an *Oncorhynchus* GMAV (versus the use of individual SMAVs for *Oncorhynchus* species) on the magnitude of the 6PPD-q screening value (Table 4-1).

The additional analysis presented below used a GMAV for *Oncorhynchus* in the FAV and FAV/2 (screening value) calculation. The additional analysis presented here is solely intended to support the screening value for acute exposures to 6PPD-q in freshwater through an evaluation of the influence of data variation on the screening value derivation process.

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

Purpose of Additional Analysis	Details of Additional Analysis	Calculated Acute Water Column Concentration for Additional Analysis (ng 6PPD-q/L)	*Oncorhynchus* References Used in both Analyses
To examine the effect of calculating an *Oncorhynchus* GMAV on the magnitude of the 6PPD-q screening value	An *Oncorhynchus* GMAV of 3.379 µg 6PPD-q/L was calculated from the four SMAVs (of 0.06134, 0.8087, >40 and 65.68 µg 6PPD-q/L) and used in the calculation in place of the SMAVs.	130	Brinkmann et al. (2022); Di et al. (2022); Greer et al. (2023a); Lo et al. (2023); Nair et al. (2023); Tian et al. (2022)
Screening Value for Acute Exposures of 6PPD-q in Freshwater using *Oncorhynchus* SMAVs		**11 ng/L**	

For this analysis, an *Oncorhynchus* GMAV of 3.379 µg 6PPD-q/L was used instead of four separate SMAVs for this genus as was done for the screening value (i.e., 0.06134, 0.8087, >40 and 65.68 µg 6PPD-q/L; ranging over four orders of magnitude, and inconclusive for *O. nerka* (non-definitive value) as summarized in Section 3.1.1.3.1). The FAV and screening value (FAV/2) calculated via this additional analysis averaging across all *Oncorhynchus* species

yielded a value of 0.2610 and 0.13 µg/L, respectively. This alternate screening value would underestimate risk to the most sensitive salmonid species (i.e., coho salmon) and be less protective. The genus-based screening value is over two times greater than the coho salmon SMAV (0.06134 µg/L). This genus-based FAV calculation approach would also be inconsistent with the *Guidelines*, which recommends against averaging values varying over one order of magnitude (a factor of 10). Based on this additional analysis, the EPA decided to retain the use of the separate SMAVs for coho salmon, rainbow trout, sockeye salmon and chinook salmon (as presented in Section 3.1.1.3.1), in development of the screening value.

4.2 Qualitative Study Summaries

Several studies were identified as not meeting the EPA's data quality considerations for inclusion in the quantitative data set for the screening value derivation. These studies were used qualitatively as supporting information and provide additional evidence of the observed toxicity and effects of 6PPD-q on other freshwater aquatic species. The key studies with apical endpoints (e.g., acute mortality) that were used qualitatively in the derivation of the 6PPD-q screening value are summarized below. These studies are sorted according to test species that are within the same species and genus sensitivity rank as those that make up the acute screening value dataset (e.g., the species coho salmon, genus *Salvelinus,* species rainbow trout, and genus *Danio* presented first, followed by other salmonids, other fish, and then invertebrates). NOEC and LOEC values were provided in several of the study summaries to allow comparison to the acute toxicity values summarized in the Effects Analysis section (see Section **Error! Reference source not found.**). A small subset of key studies with toxicity values summarized in this section were included in the acute screening value dataset to fill three missing MDRs (i.e., planktonic and benthic crustaceans and a third family in the phylum Chordata – refer to Section

3.1), but were not among four most sensitive organism groups that drive the numeric calculation of the acute screening value. These qualitative studies used to fulfill the MDRs were however included in the number of taxa (N) represented in the screening value calculation. Several qualitative studies were not used due to more significant shortcomings with the tests, such as single concentration tests resulting in no observed effects. The results of all of these studies and the rationale for why a study was not considered acceptable for quantitative use are included within each study-specific summary. These studies were qualitatively considered to ensure the water column screening value is protective of aquatic organisms, based on available data.

Figure 4-1 below includes both the quantitative (filled symbols) data that are summarized in Section 3.1.1 and the qualitative used and other qualitative (open symbols) data that are summarized below. These values are shown to provide context to the protectiveness of the screening value for 6PPD-q. The presentation of both the quantitative and qualitative 6PPD-q data demonstrates two key points regarding the protectiveness of the screening value presented in this document. First, Figure 4-1 shows that salmonids (genera of *Oncorhynchus*, *Salvelinus*, and *Salmo* in particular) appear to be the most sensitive genera to acute exposures of 6PPD-q. Second, the presentation of both the quantitative and qualitative data displays the relative sensitivity of other aquatic organisms and the inherent protectiveness of this screening value of aquatic life beyond salmonids.

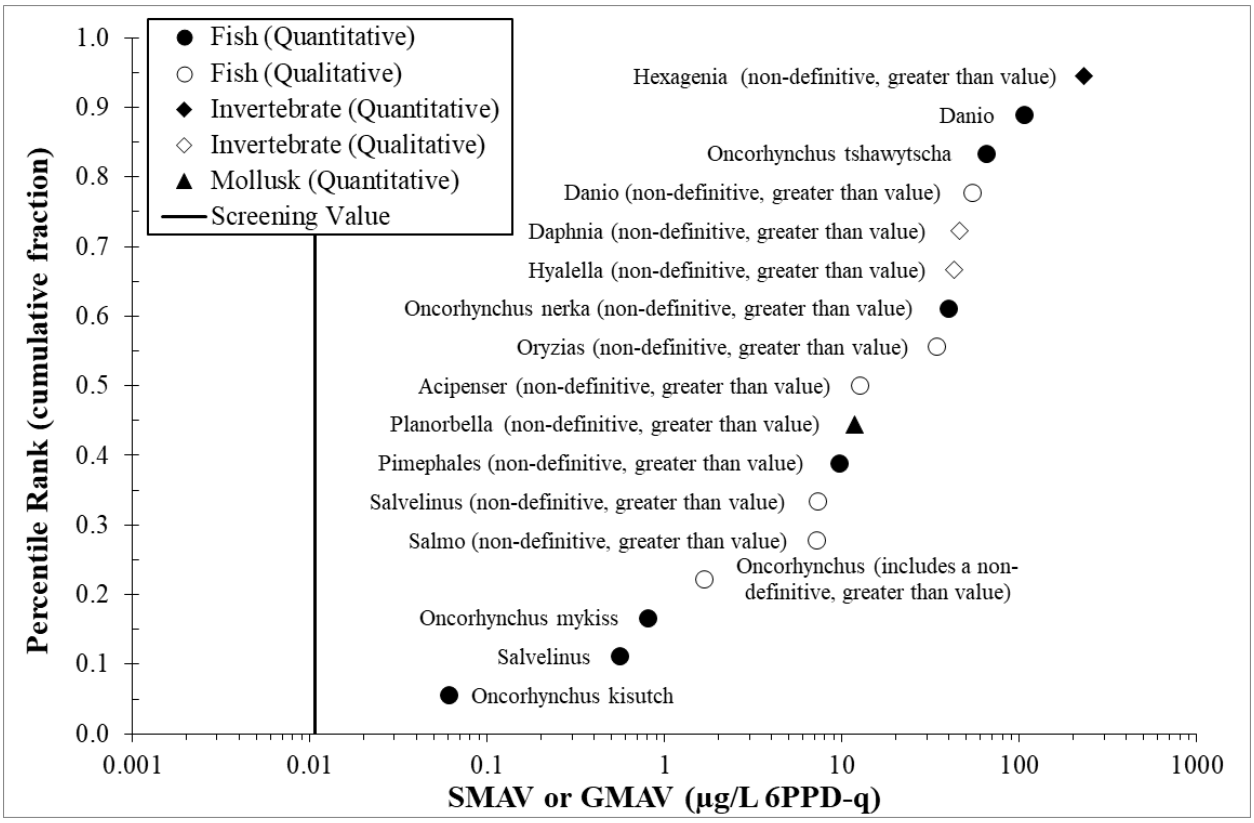

Figure 4-1. Screening Value and Aquatic Life Sensitivity Distribution for 6PPD-q in Freshwater.

The quantitative studies were summarized above in Section **Error! Reference source not found.** 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 Screening Value for 6PPD-q in Freshwater.
Ensuing study summaries are below. Underlined text highlights critical deficiencies of a study.

Taxonomic Group	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	Salmonidae	Coho salmon (juvenile, 0-2 yr), *Oncorhynchus kisutch*	S, U	24 hr	6PPD-q ~98%	0.38 – 7.5	LC_{50}	0.79	Duration too short (24 hours)[d]; limited test details; <u>only four fish per treatment[c]</u>; Despite the high biomass loading[b] there was 0% mortality in controls and D.O. saturation > 60% (ammonia not reported)	Tian et al. (2021)
Fish	Salmonidae	Coho salmon (embryo), *Oncorhynchus kisutch*	S, U	4 x 24 hr pulses	6PPD-q ~97.26%		LOEC (mortality at 6 dph)	7.22	24 hour pulsed exposures over a 14-day exposure period	Greer et al. (2023b)
Fish	Salmonidae	Japanese salmon (juvenile, <1 yr), *Oncorhynchus masou ssp. masou*	R, M	96 hr	6PPD-q >95%	5.36	NOEC (mortality)	>3.5	Only one exposure concentration resulting in no definitive effect value, <u>a greater than low value[e]; only four fish per treatment[c]</u>	Hiki and Yamamoto (2022)

Taxonomic Group	Family	Species (lifestage)	Method[a]	Test Duration	Chemical/ Purity	Biomass Loading Rate (g/L)[b]	Effect	Reported Effect Concentration (µg/L)	Deficiencies	Reference
Fish	Salmonidae	Southern Dolly Varden (juvenile, <1 yr), *Salvelinus curilus*	R, M	96 hr	6PPD-q >95%	2.88	NOEC (mortality)	> 3.8	Only one exposure concentration resulting in no definitive effect value, a greater than low value[e]; only four fish per treatment with no replication[c]	Hiki and Yamamoto (2022)
Fish	Salmonidae	Artic char (juvenile, ~3 yr), *Salvelinus alpinus*	R, M	96 hr	6PPD-q 97%	0.47	LC_{50}	>14.2	Only one exposure concentration resulting in no definitive effect value, a greater than low value[e]	Brinkmann et al. (2022)
Fish	Cyprinidae	Zebrafish (embryo, <3 hpf), *Danio rerio*	R, M	96 hr	6PPD-q 83.6%	NA	LC_{50}	>54	Only one exposure concentration resulting in no definitive effect value, a greater than low value[e]; Zebrafish embryo study (biomass loading not applicable)	Hiki et al. (2021)
Fish	Cyprinidae	Zebrafish (adult, 4 mo), *Danio rerio*	S, M	12 hr	6PPD-q 98.0%	0.3	NOEC (swimming speed and distance)	1,000	Duration too short[d]; non-apical effect endpoint	Ji et al. (2022)
Fish	Cyprinidae	Zebrafish (embryo, 8 hpf), *Danio rerio*	R, U	112 hr	6PPD-q >98%		NOEC (mortality)	1,200	Atypical test duration	Zhang et al. (2023)

Taxonomic Group	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 Fish Genera without Quantitative Data										
Fish	Salmonidae	Atlantic salmon (alevin), *Salmo salar*	S, M	48 hr	6PPD-q Not reported	1.11 – 1.443	LC_{50}	>5.75	Duration too short[d]; Only one exposure concentration resulting in no definitive effect value, a greater than low value[e]; Despite the high biomass loading[b] there was 0% mortality and D.O. saturation > 60% (ammonia not reported)	Foldvik et al. (2022)
Fish	Salmonidae	Brown trout (alevin), *Salmo trutta*	S, M	48 hr	6PPD-q Not reported	0.76 – 0.988	LC_{50}	>8.98	Duration too short[d]; Only one exposure concentration resulting in no definitive effect value, a greater than low value[e]; Despite the high biomass loading[b] there was 0% mortality and D.O. saturation > 60% (ammonia not reported)	Foldvik et al. (2022)

Taxonomic Group	Family	Species (lifestage)	Method[a]	Test Duration	Chemical/ Purity	Biomass Loading Rate (g/L)[b]	Effect	Reported Effect Concentration (µg/L)	Deficiencies	Reference
Fish	Acipenseridae	White sturgeon (juvenile, ~4.5 yr), *Acipenser transmontanus*	R, M	96 hr	6PPD-q 97%	1.32	LC_{50}	>12.7	Only one exposure concentration resulting in no definitive effect value, a greater than low value[e]; only 6 organisms per exposure concentration[c]; Despite the high biomass loading[b] there was 0% mortality and D.O. saturation > 60% (ammonia not reported)	Brinkmann et al. (2022)
Fish	Adrianichthyidae	Medaka (41 d), *Oryzias latipes*	R, M	96 hr	6PPD-q 83.6%	Not reported	LC_{50}	>34	Only one exposure concentration resulting in no definitive effect value, a greater than low value[e]	Hiki et al. (2021)
Qualitative Invertebrate Data – Used to Demonstrate that Aquatic Invertebrates Are Not Sensitive										
Planktonic crustacean	Daphniidae	Cladoceran (<24 hr), *Daphnia magna*	S, M	48 hr	6PPD-q 83.6%	NA	EC_{50} (death/ immobility)	>46	Only one exposure concentration resulting in no definitive effect value, a greater than low value[e]	Hiki et al. (2021)

Taxonomic Group	Family	Species (lifestage)	Method[a]	Test Duration	Chemical/ Purity	Biomass Loading Rate (g/L)[b]	Effect	Reported Effect Concentration (µg/L)	Deficiencies	Reference
Benthic crustacean	Hyalellidae	Amphipod (3-5 d), *Hyalella azteca*	R, M	96 hr	6PPD-q 83.6%	NA	LC_{50}	>43	Only one exposure concentration resulting in no definitive effect value, a greater than low value[e]	Hiki et al. (2021)

[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 (U.S.EPA 2016b).

[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 (U.S.EPA 2016b).

[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 (U.S.EPA 2016b).

[e] Consistent with past practice, a decision rule was applied to the 6PPD-q toxicity data as follows: i.e., "greater than" (>) high toxicity values and "less than" (<) low toxicity values were included; other non-bounded values were not (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 Two Most Sensitive Genera with Quantitative Data

4.2.1.1.1 Genus Oncorhynchus

Tian et al. (2021) evaluated the 24-hour toxicity of 6PPD-q (approximately 98% purity) to coho salmon (*O. kisutch*). Individual aquaria contained 30 L of test solution. This biomass loading rate (between 0.38 g/L and 7.5 g/L) may have exceeded the EPA's 850 Ecological Effects Test Guidelines in some tanks (0.8 g/L; U.S.EPA 2016b). Twenty-four-hour toxicity tests were performed using eight juvenile fish per concentration across 10 measured concentrations (0 [control], ~0.1 [control], ~0.2, ~0.3, ~0.5, ~0.8, ~1, > 1- < 2, ~2 µg/L, ~3, ~4 and ~5 µg/L). A solvent control (material and amount by volume not provided) and a positive control (250 mg/L TWP leachate) were additionally included. Each concentration was replicated twice. No mortality was observed in the control, and 100% mortality was observed in the highest exposure concentration, resulting in a 24-hour LC_{50} of 0.79 µg/L for 6PPD-q. The supplemental materials state a LC_{50} of 1.46 µg/L. The EPA reached out to the study authors to inquire about the differences between the values reported. The 24-hour LC_{50} of 0.79 µg/L 6PPD-q represents the measured data and the LC_{50} of 1.46 µg 6PPD-q/L in the supplemental materials represents the nominal concentrations. The EPA considered the 24-hour LC_{50} of 0.79 µg/L 6PPD-q from measured concentrations acceptable for qualitative use because the exposure concentration was only 24 hours, as opposed to the recommended test duration of 96-hours in the EPA's 850 Ecological Effects Test Guidelines (U.S.EPA 2016b) and there appear have been issues with biomass loading. The 24-hour LC_{50} of 0.79 µg 6PPD-q/L originally stated in the paper was amended in an errata to the original article, which stated that the latest LC_{50} was 0.095 µg 6PPD-q/L with the use of a commercial standard of 6PPD-q. This later value was presented in greater detail in Tian et al. (2022) (see Section 3.1.13.1.1.1). The two concerns noted above together

with the change in the LC_{50} value presented in the paper resulted in the qualitative use of the original study and corresponding LC_{50} value of 0.79 µg 6PPD-q/L.

Greer et al. (2023b) conducted a 14-day exposure consisting of four 24-hour pulsed measured, static acute test of 6PPD-q (97.26% purity, obtained from HPC Standards) with the coho salmon, *O. kisutch*. Embryos were exposed to 24-hour pulses of 6PPD-q or solvent carrier control (volume not provided) twice per week until hatch (six replicate dishes, n = 120 embryos/concentration) to mimic intermittent winter rain events. Embryos were exposed to one of four pulsed exposures at nominal concentrations of 0.1, 1 or 10 µg/L 6PPD-q), plus a DMSO solvent control. After each 24-hour exposure, eggs were carefully transferred back to egg rearing trays (1 tray per treatment group) and maintained in clean flowing water. Embryos that hatched prior to the final exposure were excluded from subsequent exposures but continued to be monitored for mortality. Surviving, unhatched embryos were randomly distributed to the dishes for the next (fourth) exposure. Mortality and hatching were monitored daily throughout the experiment, with hatching defined as the complete liberation of both the head and tail from the chorion. Embryos exhibiting abnormal phenotypes were monitored for a minimum of 24 hours to ensure the embryo was not in the process of a successful hatch. The larvae assessed in the experiment were collected directly from the glass exposure dishes following the fourth exposure in which most embryos hatched to ensure that all larvae measured were less than 24 hours post-hatch. No mortality was observed in the solvent control treatment. No effects were observed in any of the test concentrations after the first 24-hour pulse. Effects at the highest test concentration started to occur after the second 24-hour exposure. The author-reported 14-day LOEC for mortality at 6 dph of eyed embryos was 7.22 µg 6PPD-q/L, which was acceptable for qualitative use, because of the atypical pulsed-exposure test design. The results of this test

occurred at higher concentrations than what was observed for this species via the quantitative test results (SMAV of 0.06134 µg/L), thus effects observed here on embryos would be protected by the acute test outcomes and the screening value.

Hiki and Yamaoto (2022) examined the acute effects of 6PPD-q (purity > 95%, Cambridge Isotope Laboratories) to the Japanese salmon, *Oncorhynchus masou ssp. Masou.* The test methodology used (static-renewal, measured exposure) was very similar to that used by the authors when testing Southern Dolly Varden trout (*Salvelinus curilus*), summarized below. Acute static-renewal measured lethality tests were performed according to the OECD Test Guideline 203 with slight modifications that were determined to be inconsequential. No lethality or abnormal behavior of fish was observed during the exposure duration. Thus, the 96-hour LC_{50} was >3.5 µg/L 6PPD-q (based on the time weighted average measured concentration). This study was deemed qualitatively acceptable because there was only one concentration tested, which resulted in a greater than low value, and only four fish per treatment.

4.2.1.1.2 Genus Salvelinus

Hiki and Yamamoto (2022) also evaluated the acute toxicity of 6PPD-q to the Southern Dolly Varden trout, *S. curilus*, using a test methodology (static-renewal, measured exposure) that was similar to the quantitative study on whitespotted char (*Salvelinus leucomaenis ssp. pluvius*) summarized in Section 3.1.1.2. The 6PPD-q (purity > 95%, Cambridge Isotope Laboratories) acute static-renewal measured lethality tests were performed according to the OECD Test Guideline 203 with slight modifications that were determined to be inconsequential. No lethality or abnormal behavior of fish was observed during the exposure duration. The 96-hour LC_{50} was > 3.8 µg/L 6PPD-q (based on the time weighted average measured concentration). This study was deemed qualitative because there was only one concentration tested. Additionally, there were only four fish per treatment whereas the EPA'S 850 Ecological Effects Test Guidelines

states that a minimum of seven fish are required and that 10 fish and two replicates per treatment level is preferred to obtain a more statistically accurate representation of the C-R curve (U.S.EPA 2016b). This author-reported LC_{50} of >3.8 µg 6PPD-q/L was used qualitatively as supporting information and indicates that Southern Dolly Varden would be protected by the acute freshwater screening value for 6PPD-q.

The sensitivity of the Artic char, *Salvelinus alpinus*, to 6PPD-q was evaluated by **Brinkmann et al. (2022)** via a 96-hour static-renewal, measured toxicity test. The 6PPD-q purity was not reported in paper. The EPA's personal communication with study authors provided a purity of 97%, purchased from Toronto Research Chemicals. No mortalities were observed at the measured exposure concentration of 14.2 µg 6PPD-q/L after 96 hours. The resultant 96-hour LC_{50} is >14.2 µg 6PPD-q/L. This test was deemed qualitative since there was only one exposure concentration.

4.2.1.2 Qualitatively Acceptable Acute Data for Other Fish Species Used as Supporting Information

4.2.1.2.1 Genus Salmo

Foldvik et al. (2022) investigated the acute toxicity of 6PPD-q (purity not reported, purchased from Cambridge Isotope Laboratories) to the Atlantic salmon, *Salmo salar* in a static, measured exposure. No mortality or abnormal behavior was observed during the experiment. The authors reported a LC_{50} of >5.75 µg 6PPD-q/L. However, this study was classified as qualitative because exposure occurred for only 48 hours (not the standard 96-hours required) with a single test concentration. The acute screening value of 0.011 µg/L would be protective of this species based on these results.

Foldvik et al. (2022) also evaluated the static, measured acute sensitivity of *Salmo trutta* (brown trout) to 6PPD-q (purity not reported, purchased from Cambridge Isotope Laboratories).

Test procedures were essentially the same as used by the authors for *S. salar*. No mortality or abnormal behavior was observed during the experiment. The authors reported a LC_{50} of >8.98 µg 6PPD-q/L. However, this study was also classified as qualitative because the exposure was 48 hours with a single test concentration.

4.2.1.2.2 Acipenser transmontanus

A third species evaluated by **Brinkmann et al. (2022)** for acute sensitivity to 6PPD-q, was the white sturgeon, *Acipenser transmontanus* via a static-renewal, measured exposure. Although chemical purity was not reported in paper, the EPA's personal communication with study authors confirmed that 6PPD-q with purity of 97% and purchased from Toronto Research Chemicals was used. The test procedures were essentially the same as those used by the authors when testing the Artic char, *S. alpinus*. Fish were exposed to only one nominal concentration (20 µg/L) that could be achieved using the limited amount of chemical available and that was nearing water solubility, while still being environmentally relevant. Test organisms were exposed in 700 L glass-fiber Min-o-Cool tanks containing 500 L of test solution at 12 ± 1 °C for 96 hours under static-renewal conditions. A total of 12 fish were exposed (three replicate tanks each for the control and 6PPD-q treatment with two fish per tank). No mortalities were observed at the measured exposure concentration of 12.7 µg/L after 96 hours. The resultant 96-hour LC50 is >12.7 µg/L. This test was deemed qualitative since there was only one exposure concentration and there were too few test organisms per treatment compared to the EPA's 850 Ecological Effects Test Quality Guidelines. The results from this qualitative test were used to fulfill the missing MDR for a third Family in the Phylum Chordata. The relative insensitivity of this test result, as deficient as the overall test was for said reasons, indicates the acute screening value would be protective of white sturgeon.

4.2.1.2.3 Danio rerio

Hiki et al. (2021) performed a 96-hour static-renewal, measured test 6PPD-q (83.6 purity, synthesized in the laboratory) with the zebrafish, *Danio rerio*. The acute toxicity test followed OECD guideline number 236 (OECD 2013). 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 test concentration resulting in a 96-hour LOEC of >54 µg 6PPD-q/L. It is considered acceptable for qualitative use because there was only one test concentration, which resulted in a low greater than LOEC (U.S.EPA 2013).

Ji et al. (2022) performed a 12-hour static measured test of 6PPD-q (98.0% purity, obtained from Jiaxuan Biotechnology Co., Ltd., Hengyang, China) with the zebrafish, *D. rerio*. No statistically significant differences ($P > 0.05$) in swimming velocity or distance were observed during the experiment, resulting in a 12-hour LOEC of >1,000 µg 6PPD-q/L. The test was considered qualitative because it is of insufficient duration (12 hours as opposed to the 96 hours preferred in the EPA's 850 Ecological Effects Test Guidelines (U.S.EPA 2016b) or 24 hours that is commonly used in other 6PPD-q toxicity studies summarized in this document) for an acute toxicity test and is based on a non-apical (and relatively insensitive, in this test) behavioral endpoint.

Zhang et al. (2023) conducted a 112-hour static-renewal, unmeasured acute test of 6PPD-q (>98% purity, Toronto Research Chemicals, Toronto, Canada) with the zebrafish, *Danio rerio*. At 120 hpf, the accumulated malformation and mortality was calculated using triplicates. The author-reported 112-hour mortality NOEC was 1,200 µg 6PPD-q/L, which was acceptable only for qualitative use due to atypical test duration (in this case longer than what was commonly used in other 6PPD-q toxicity studies summarized in this document and longer than what is

recommended in EPA's 850 Ecological Effects Test Guidelines, which specify that acute toxicity tests on fish should have at least 72 hours of exposure and recommend 96 hour exposures).

4.2.1.2.4 Oryzias latipes

Hiki et al. (2021) performed a 96-hour static-renewal, measured test of 6PPD-q (83.6% purity, synthesized in the laboratory) with the Japanese medaka, *Oryzias latipes*. The acute toxicity test followed OECD guideline number 203 (OECD 2019). No mortality was observed in the control or test concentration resulting in a 96-hour LC_{50} of >34 µg/L. It was considered acceptable for qualitative use because there was only one test concentration. The data from this qualitative test could also be used to fulfill the missing MDR for a third Family in the Phylum Chordata, but the value for white sturgeon was used instead because white sturgeon are more closely related to salmonids versus Japanese medaka, a cyprinid fish species. The relative insensitivity of this test result indicates the screening is protective of this species.

4.2.1.3 Qualitatively Acceptable Acute Data for Invertebrate Species Used as Supporting Information

4.2.1.3.1 Daphnia magna

Hiki et al. (2021) performed a 48-hour static measured test of 6PPD-q (83.6% purity, synthesized in the laboratory) with the cladoceran, *Daphnia magna*. The acute toxicity test followed OECD guideline number 202 (OECD 2004). No mortality was observed in the control or treatment concentration, resulting in a 48-hour EC_{50} of > 46 µg 6PPD-q/L. This value was considered acceptable for qualitative use because there was only one test concentration and this test resulted in a greater than low value, which generally should not be used quantitatively (U.S. EPA 2013). Nevertheless, the result was deemed useful to fulfill the missing MDR for a planktonic crustacean.

4.2.1.3.2 Hyalella azteca

Hiki et al. (2021) performed a 96-hour static-renewal measured test of 6PPD-q (83.6% purity, synthesized in the laboratory) 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 test concentration was 0%, resulting in a 96-hour LOEC of > 43 µg 6PPD-q/L. The test was considered acceptable for qualitative use because there was only one test concentration and this test resulted in a greater than low value, which generally should not be used quantitatively (U.S. EPA 2013). Nevertheless, the result was deemed useful to fulfill the missing MDR for a benthic crustacean.

4.2.1.4 Qualitatively Acceptable Freshwater Chronic Data

4.2.1.4.1 Megalonaias nervosa

Prosser et al. (2023) conducted an 8-day static-renewal, measured test of 6PPD-q (>99.8% purity, purchased from Toronto Research Chemicals Inc., Toronto, ON, Canada) with the washboard mussel, *Megalonaias nervosa*. Control organism survival was 100% for both tests in the water only and solvent controls. The 8-day NOEC for mortality was 11.4 µg 6PPD-q/L for Test A, and 17.9 µg 6PPD-q/L for Test B. Data from both tests were acceptable only for qualitative use due to atypical test duration of eight days and too few organisms per exposure treatment (n=4). Based on the results from this test, the washboard mussel would be protected from acute mortality by the acute screening value of 0.011 µg/L.

4.3 Effects on Aquatic Plants

The very limited available data for aquatic plants and algae were reviewed to determine if aquatic plants were likely to be more sensitive than aquatic animals to aqueous 6PPD-q exposure. Toxicity values for freshwater plants were well above the freshwater acute freshwater screening value. Effect concentrations for freshwater algae were available for one species (green

algae, *Chlamydomonas reinhardtii*) with a 72-hour LOEC of 250 µg 6PPD-q/L (see below), which is greater than freshwater chronic values for animal species: *Daphnia magna* NOEC of 30.2 µg 6PPD-q/L and 45-day LC_{50} for *Salvelinus namaycush* of 0.39 µg 6PPD-q/L (see Section 3.1.3). The plant LOEC was also greater than all of the freshwater acute SMAVs. Therefore, it was not necessary to develop a screening value based on the toxicity of 6PPD-q to aquatic plants. The 6PPD-q screening value for freshwater is expected to be protective of freshwater plants.

4.3.1 Plant Study Summaries

4.3.1.1 *Chlamydomonas reinhardtii*

Wu et al. (2023) evaluated the 72-hour toxicity 6PPD-q with the green algae, *Chlamydomonas reinhardtii* in a static, unmeasured exposure. At test termination, microalgae were collected by centrifugation at 13,000 rpm for 5 min for further analysis. The author-reported 72-hour population growth rate LOEC was 250 µg 6PPD-q/L, which was acceptable for quantitative use.

4.4 Protection of Threatened and Endangered Species

Although the 6PPD-q freshwater acute aquatic life screening value dataset is not extensive, it 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. Summaries are provided in this document describing the available 6PPD-q toxicity data for listed species indicating that the 6PPD-q freshwater acute screening value is expected to be protective of the freshwater life stages of these listed anadromous species, based on available scientific data.

4.4.1 Quantitative Toxicity Data for Listed Species

Quantitative freshwater acute toxicity test data evaluating the effects of 6PPD-q on threatened and endangered freshwater species are available for coho salmon (*Oncorhynchus*

kisutch), rainbow trout for steelhead salmon (*Oncorhynchus mykiss*), and Chinook salmon (*Oncorhynchus tshawytscha*). Coho salmon is the most acutely sensitive species to 6PPD-q, with a SMAV of 0.06134 µg/L 6PPD-q (Greer et al. 2023a; Lo et al. 2023; Tian et al. 2022). The coho salmon SMAV is 5.6 times greater than the acute screening value of 0.011 µg/L. The rainbow trout SMAV is 0.8087 µg/L (Brinkman et al. 2022; Di et al. 2022; Nair et al. 2023), and the Chinook salmon SMAV is 65.68 µg/L (Greer et al. 2023a), which are 74 and 5,971 times greater than the acute screening value, respectively. There are no quantitative freshwater chronic toxicity data, or quantitative estuarine/marine acute or chronic data, for endangered or threatened aquatic species.

4.4.2 Qualitative Toxicity Data for Listed Species

Qualitative freshwater toxicity data evaluating the effects of 6PPD-q were available for several endangered or threatened aquatic species. The washboard mussel (*Megalonaias nervosa*) is not federally listed but is listed as endangered in Minnesota (MDNR 2013) and Ohio (ODNR 2022). Prosser et al. (2023) reported two sub-chronic eight-day NOECs of 11.4 µg/L and 17.9 µg/L for mortality. Brinkman et al. (2022) reported a 96-hour acute LC50 of >12.7 µg/L for white sturgeon (*Acipenser transmontanus*). For coho salmon (*O. kisutch*), Tian et al. (2022) reported a 24-hour acute LC_{50} of 0.79 µg/L, and Greer et al. (2023b) reported a LOEC for mortality of 7.22 µg/L following four 24-hour pulsed doses over 14 days. Finally, Foldvik et al. (2022) reported a 48-hour acute LC_{50} of >5.75 µg/L for Atlantic salmon (*Salmo salar*). All of these qualitative effect levels were substantially greater than the acute screening value of 11 ng/L. There were no qualitative freshwater chronic toxicity data, or qualitative estuarine/marine acute or chronic data, for endangered or threatened aquatic species.

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Appendix A Quantitative Acute Freshwater Toxicity Data

A.1 Summary Table of Acceptable Quantitative Freshwater Acute 6PPD-q Toxicity Studies

Values used in SMAV calculation are highlighted in bold.

Species (lifestage)	Method[a]	Test Duration	Chemical / Purity	pH	Temp. (°C)	Hardness (mg/L as CaCO3)	Effect	Author Reported Effect Conc. (µg/L)	EPA Adjusted / Calculated LC_{50} (µg/L)	Species Mean Acute Value (µg/L)	Reference
File ramshorn snail (embryo), *Planorbella pilsbryi*	S, M	24 hr	6PPD-q >99.8%	7.1-7.6	24-25	105.5	LC50	**>11.7**	-	>11.7	Prosser et al. (2023)
Mayfly (larva), *Hexagenia sp.*	S, M	96 hr	6PPD-q >99.8%	6.5-7.5	23	105.5	LC50	**>232.0**	-	>232.0	Prosser et al. (2023)
Coho salmon (juvenile, >1 yr), *Oncorhynchus kisutch*	S, M	24 hr	6PPD-q 98.8%	7.6-8.0	10.0-13	-	LC50	0.095	**0.07752**	-	Tian et al. (2022)
									0.09216	-	
Coho salmon (3 week post swim up), *Oncorhynchus kisutch*	S, M	24 hr	6PPD-q 97.26%	6.8-7.3	13.8	89.8	LC50	0.041	**0.0363**	-	Lo et al. (2023)
Coho salmon (189-d old, 1.95g), *Oncorhynchus kisutch*	F, M	24 hr	6PPD-q 97.5%	-	8.0	-	LC50	0.0804[b]	**0.0546**	0.06134	Greer et al. (2023a)
Rainbow trout (juvenile, ~2 yr), *Oncorhynchus mykiss*	R, M	96 hr	6PPD-q 97%	8.35	12.8	132	LC50	**1.00**	-	-	Brinkmann et al. (2022)
Rainbow trout, *Oncorhynchus mykiss*	R, M	96 hr	6PPD-q ≥98%	-	16	-	LC50	2.26	**1.786**	-	Di et al. (2022)
Rainbow trout (juvenile. 2 mo), *Oncorhynchus mykiss*	S, M	96 hr	6PPD-q 95%	-	15	-	LC50	0.64	**0.2961**	0.8087	Nair et al. (2023)

Species (lifestage)	Method[a]	Test Duration	Chemical / Purity	pH	Temp. (°C)	Hardness (mg/L as CaCO3)	Effect	Author Reported Effect Conc. (µg/L)	EPA Adjusted / Calculated LC_{50} (µg/L)	Species Mean Acute Value (µg/L)	Reference
Chinook salmon (3 week post swim up), *Oncorhynchus tshawytscha*	S, M	24 hr	6PPD-q 97.26%	6.7-7.0	13.6	102	LC50	>67.31	>53.85[c]	-	Lo et al. (2023)
Chinook salmon (~582-d old, 12.1 g), *Oncorhynchus tshawytscha*	F, M	24 hr	6PPD-q 97.5%	-	10.0	-	LC50	82.1[b]	**65.68**	65.68	Greer et al. (2023a)
Sockeye salmon (~625-d old, 6.46 g), *Oncorhynchus nerka*	F, U	24 hr	6PPD-q 97.5%	-	10.0	-	LC50	>50[b]	**>40.00**	>40.00	Greer et al. (2023a)
Brook trout (juvenile, ~1 yr), *Salvelinus fontinalis*	S, M	24 hr	6PPD-q 97%	6.74	10.3	131	LC50	**0.59**	-	0.59	Brinkmann et al. (2022)
Whitespotted char (juvenile, <1 yr), *Salvelinus leucomaenis ssp. pluvius*	R, M	96 hr	6PPD-q >95%	7.5	14.4	68	LC50	0.80	**0.5709**	0.5709	Hiki and Yamamoto (2022)
Lake trout (juvenile, 8 week post hatch), *Salvelinus namaycush*	R, M	96 hr	6PPD-q 97%	8.23	10	92.67	LC50	0.50	**0.5186**	0.5186	Roberts et al. 2024
Zebrafish (embryo), *Danio rerio*	R, U	96 hr	6PPD-q >98%	6.81	26	-	LC50	132.9	**106.3**	106.3	Varshney et al. (2022)
Fathead minnow (adult), *Pimephales promelas*	R, M	96 hr	6PPD-q 97%	-	22	-	LC50	**>9.65**	-	>9.65	Anderson-Bain et al. (2023)

[a] S=Static, R=static-renewal, M= measured, U=unmeasured

[b] Exposures were conducted by concentration on a daily basis (2-3 concentrations, one tank each). Replication was done across different days, and the number of replicates varied across concentrations. Both nominal and measured concentrations were used to fit the C-R curve for LC_{50}s. Concentrations were not measured in sockeye salmon exposures.

[c] Not used in SMAV calculation because definitive value is available.

A.2 Detailed 6PPD-a 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-q with most summaries being grouped based on sensitivity at the genus level, with the exception of *Oncorhynchus* which is grouped by sensitivity at the species level. 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. 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 taxon: *Oncorhynchus kisutch* (coho Salmon - salmonid)

Lo et al. (2023) conducted a 24-hour static, measured acute test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, 97.26% purity, obtained from HPC Standards, Atlanta, CA) with the coho salmon, *Oncorhynchus kisutch*. Juvenile coho salmon (3 weeks post-swim up with an average body weight of 0.433 g wet weight) used for testing were obtained from Chehalis River Hatchery (Agassiz, BC). After feeding for one week, fish were transported to the Pacific Science Enterprise Centre (PSCE) and then reared for two weeks in 125 L flow-through glass-fiber tanks prior to 6PPD-q exposure. Rearing conditions were consistent with Environment Climate Change Canada's *Biological Test Method: Acute Lethality Using Rainbow Trout* (ECCC 1990). Well water was used for culturing and exposures. Stock solutions of 6PPD-q were prepared using absolute ethanol, resulting in a final solvent concentration of 0.01% (v/v). These stock solutions were prepared 24 hours before exposure. For

the coho salmon exposures, a single stock solution was created, and subsequent stock solutions were made by serial dilution. Test chambers were 20 L glass tanks with 18 L of test solution and 14 fish per replicate tank, which resulted in a biomass loading rate of 0.337 g/L. The 6PPD-q exposures consisted of five nominal concentrations of 11.9, 21.4, 38.6, 69.4, 125 ng 6PPD-q/L and separate well water and solvent (0.01% ethanol) controls, with four replicates each. All exposures were conducted under static conditions with continuous aeration, a photoperiod of 16:8 hours (light:dark) and light intensity of 100 – 500 lux. Fish were not fed during testing. Average water quality conditions based on measurements taken at the initiation and termination of the test consisted of a water temperature of 13.8 ± 0.3, dissolved oxygen (D.O.) > 86%, pH between 6.8 – 7.3, and conductivity of 89.8 mg/L. Test concentrations of 6PPD-q were measured in two replicates from each of the treatment groups at the initiation and termination of the test (the same replicates at each sampling event). Average concentrations of 6PPD-q at the test initiation deviated 22.8 ± 16.9% (3.3 – 49.3%) from the nominal concentration. The average loss of 6PPD-q after 24 hours of exposure was 35.2 ± 17.1%, as reported by the study authors. Survival in the well water control was 100% across all four replicates. Survival in one replicate of the solvent controls was 71.4% as fish exhibited symptoms consistent with exposure to 6PPD-q. The study authors report that a subsample from the solvent control tank with the symptomatic fish had 6PPD-q contamination (34.4 ng/L) and data from the contaminated replicate was excluded from the analysis. The remaining three solvent control replicates exhibited 100% survival. The authors noted that fish exhibited symptoms (e.g., gasping, loss of equilibrium, erratic swimming) and mortality during the fourth hour of exposure at the highest test concentration (104.7 ng/L) with 100% mortality in two of the four replicates; overall average survival in the highest test concentration was 7.1% across all four replicates. However, some

individual fish did not exhibit symptoms at any point during the 24 hours despite exposure to 6PPD-q. The author-reported 24-hour LC_{50} was 0.041 µg 6PPD-q/L, which was based on initial measured concentrations. Due to holding time losses of 6PPD-q within samples, study authors performed a degradation study. The results from the degradation study were natural-log transformed and fitted to a linear regression to determine a slope by which the measured toxicity concentration values at the time of initiation could be estimated from. 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 0.0363 µg/L 6PPD-q, which was acceptable for quantitative use.

Tian et al. (2022) evaluated the toxicity of N-(1,3-Dimethylbutyl)-N'-phenyl-p-phenylenediamine-quinone (6PPD-q, CAS# 2754428-18-5, 98.8% purity, purchased from HPC - Atlanta, GA) to juvenile coho salmon (*O. kisutch*) for 24 hours under static measured conditions. The 6PPD-q stock solution (stored at -20 ºC) was made by dissolving 5 mg of the standard in 50 mL of ethanol. Exposures were prepared by diluting various volumes of the 6PPD-quinone stock solution in 10 mL of ethanol (350-1400 ng/mL), which was then mixed with 70 L of system water. Solutions and negative solvent controls (10 mL pure ethanol per 70 L) were made within 24 hours of exposure. Juvenile coho salmon were obtained from the Puyallup Tribe of Indians, from the same stock (Diru Creek) as in a previous study (Tian et al. 2021) and from the same cohort but older and larger (>1 year old, 30-64 g). Because of the larger fish, larger exposure volumes (70 L) and fewer fish per aquarium (N = 6) were used in this study compared to Tian et al. (2021). The biomass loading rate of 2.6-5.5 g/L exceeds that recommended in the EPA's 850 Ecological Effects Test Guidelines (0.8 g/L). While the biomass loading rate was over 3.2 times higher than the 0.8 g/L rate recommended in the EPA's 850 Ecological Effects Test Guidelines (U.S.EPA 2016a), there were no signs of stress in the test (solvent) control organisms (0%

mortality and D.O. saturation >60% in the controls) based on the information provided in the paper. Ammonia concentrations were not reported in the paper, but the EPA has reached out to the study authors and is awaiting additional information. Fish were reared at Washington State University's Puyallup Research and Extension Center on a 12 h:12 h light:dark cycle in a custom recirculating water system and fed commercial food (Biovita). Fish system water was dechlorinated municipal water treated by reverse osmosis to Type 3 (< 0.25 uS/cm) in a RiOs 200 purification system (Millipore Sigma) and then reconstituted with buffered Instant Ocean salts to pH ≈7.6 and 1,300 uS/cm conductivity at 10 - 13 ºC. The LC_{50} was calculated by combining the results of four separate exposure series using different concentrations of six concentrations of 6PPD-q and a negative control. Fish were not fed during testing. Four exposure series were tested; in the first series treatments were more widely spaced, while the remaining three series were more narrowly focused within the concentration range where partial mortality was expected. Exposures were repeated in triplicate across three weeks. Water quality conditions during the test were maintained at 10 - 13 ºC water temperature, 1,170 - 1,370 µS/cm specific conductivity, pH of 7.6 - 8.0, and dissolved oxygen of > 98% of saturation. Just prior to the introduction of fish, 1 L of exposure water was sampled from each aquarium, stored on ice or refrigerated, and extracted within 24 hours for analysis. No mortality was observed in the control. There was no information in the publication about time of death or immobility in the definitive exposure as fish were sampled at 24 hours, but in range-finding experiments, the authors noted that fish in the 4.0 µg/L aquarium were symptomatic in ~40 minutes and all perished in < 2 hours. This speed was faster than any of the authors' prior observations in Tian et al. (2021). The author-reported 24-hr LC_{50} was reported as 0.095 µg 6PPD-q/L and was based on initial measured concentrations only and combining each experimental series into one C-R curve.

The EPA curve fit the data based on each experimental series (or batch, as reported in the paper). The LC_{50} values from each series that could be curve fit were used as opposed to combining all series into one curve fit. The EPA-calculated LC_{50}s (0.09693 and 0.1152 µg/L, respectively) were adjusted to lower the value by 20% in order to account for loss of 6PPD-q over the experiment duration. The adjusted EPA-calculated LC_{50}s were 0.07752 and 0.09216 µg/L 6PPD-q, which were acceptable for quantitative use.

Greer et al. (2023a) conducted a 24-hour partially-measured, flow-through acute test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, 97.5% purity, obtained from HPC Standards, Atlanta, GA) with the coho salmon, *O. kisutch*. Young-of-year coho salmon (189 days old with an average initial body weight of 1.95 g wet weight) used for testing were obtained from the Issaquah Salmon hatchery (Issaquah, WA). Fish were housed in 175 L flow-through tanks supplied with freshwater from Lake Washington, UV-treated and filtered at the US Geological Survey Western Fisheries Research Center in Seattle, WA. Water temperature was maintained at 8°C. Fish were fed ~ 2% of body weight every other day with commercial pellets during holding. 6PPD-q was dissolved in dimethyl sulfoxide (DMSO) to make a concentrated stock solution. The stock solution was further diluted in in the same holding tank water for the toxicity test. Coho salmon (n= 6/tank) were exposed to 6PPD-q in 9 L tanks containing 8 L of water supplied with constant aeration via air stone. Tanks were set inside a 275 L flow-through circular tank with a low flow of temperature-controlled dilution water to maintain water temperature (8°C). Individuals were allowed to acclimate for a minimum of 1 hour prior to exposure. Fish were not fed for 24 hours prior to exposure. To ensure thorough and rapid mixing during exposures, 2 L of fresh dilution water were vigorously poured into each tank while 1 L of the appropriate 6PPD-q stock solution was added. Fish were exposed to 6PPD-q

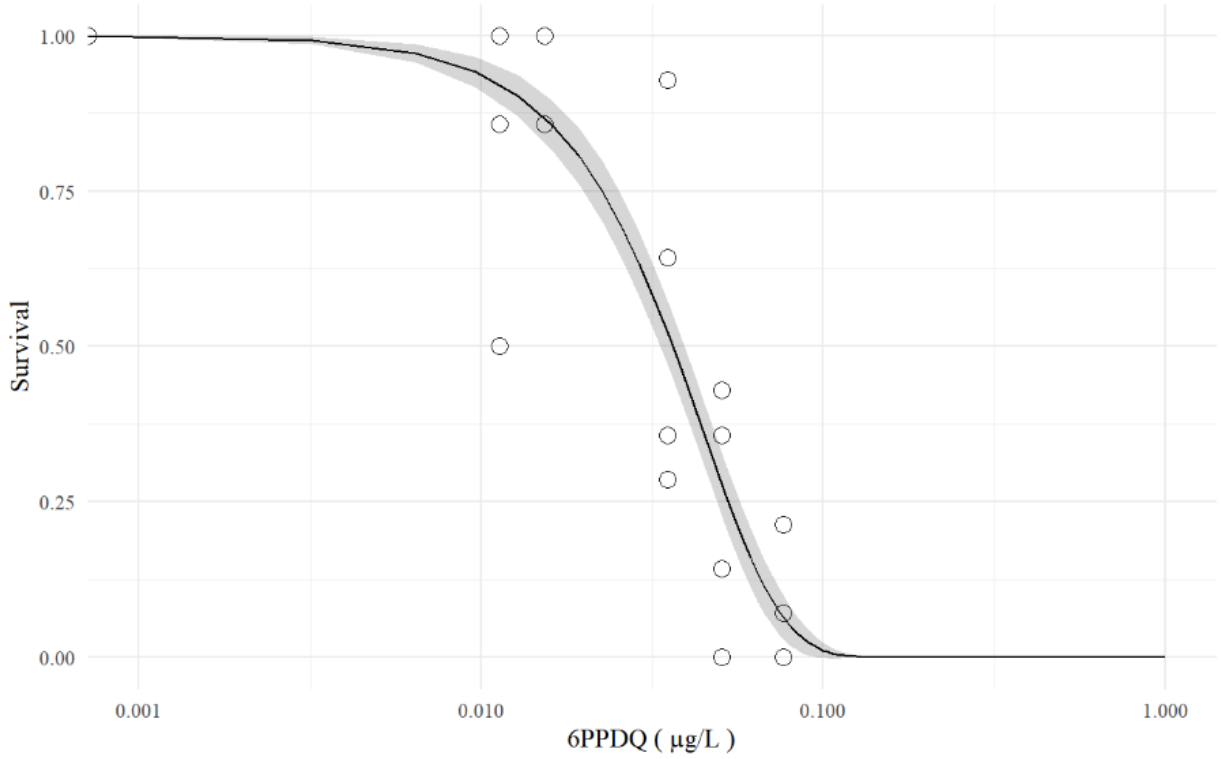
concentrations ranging from 25 to 125 ng/L (or 0.025 to 0.125 µg/L; n = 20 exposures) over the course of multiple days, with 2-3 tanks tested per day, each with a different 6PPD-q concentration. Replication was done across different days, and the number of repeated exposures varied across concentrations (e.g., exposure to 100 ng/L 6PPD-q was performed many times for coho salmon, whereas 125 ng/L was only performed twice). The approach was taken to focus on concentrations defining the slope of the curve. Both nominal and measured concentrations were used to fit the curve for the coho LC_{50}. There were five measured and 15 nominal concentration experiments total. Mortalities were monitored for 24 hours. Mass and fork length were measured for each individual. No mortality was observed in any solvent control treatments (amount of DMSO not provided). Water samples were collected from each tank within the first 30 seconds of exposure. Select samples were analyzed at the U.S. Geological Survey Organic Geochemistry Research Laboratory by a direct-inject Waters Corporation Acquity H-Class Bio UPLC/Sciex API 5500 triple quadrupole mass spectrometer (UPLC/MS/MS). Photoperiod and light intensity were not reported, nor were average water quality conditions based on measurements taken during the flow-through test. There was no information in the publication about time of death or immobility, but authors noted that all fish exhibited the suite of symptoms associated with URMS. The author-reported 24-hour LC_{50} for young-of-year coho salmon was 80.4 ng 6PPD-q/L, or 0.0804 µg/L. The author-reported value was a combination of measured and nominal concentrations. The EPA curve fit the data to calculate an LC_{50} value based on measured average concentrations only. The EPA-calculated LC_{50} was 0.0546 µg/L 6PPD-q, which was acceptable for quantitative use.

A.2.1.1 Lo et al. 2023 Concentration Response Curve – Oncorhynchus kisutch (Coho salmon)

Publication: Lo et al. 2023
Species: *Oncorhynchus kisutch*
EPA-Calculated LC_{50}: 0.0363 (0.0284 – 0.0441) µg/L
Concentration-Response Model Estimates:

Parameter	Estimate	Std. Error	t-stat	p-value
b	1.848	0.1352	13.668	$< 2.2e^{-16}$
e	0.0442	0.0017	26.34	$< 2.2e^{-16}$

Concentration-Response Model Fit:



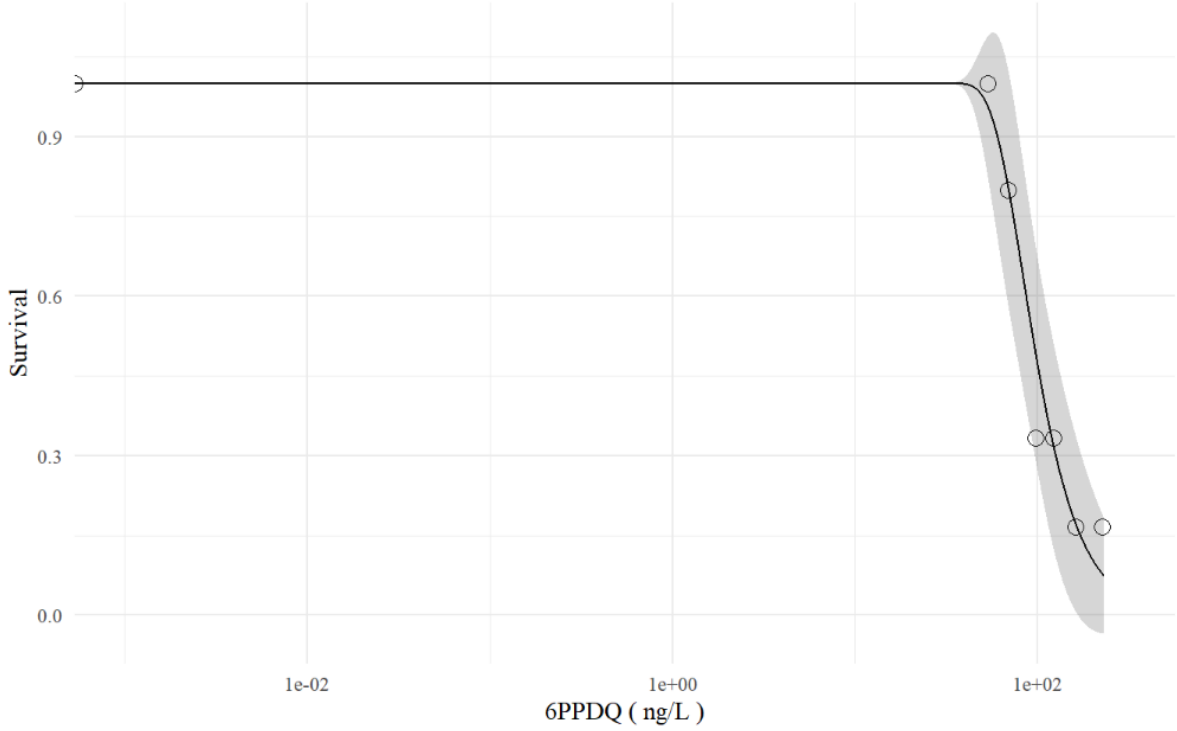
A.2.1.2 Tian et al. 2022 Concentration Response Curve – Oncorhynchus kisutch (Coho salmon)

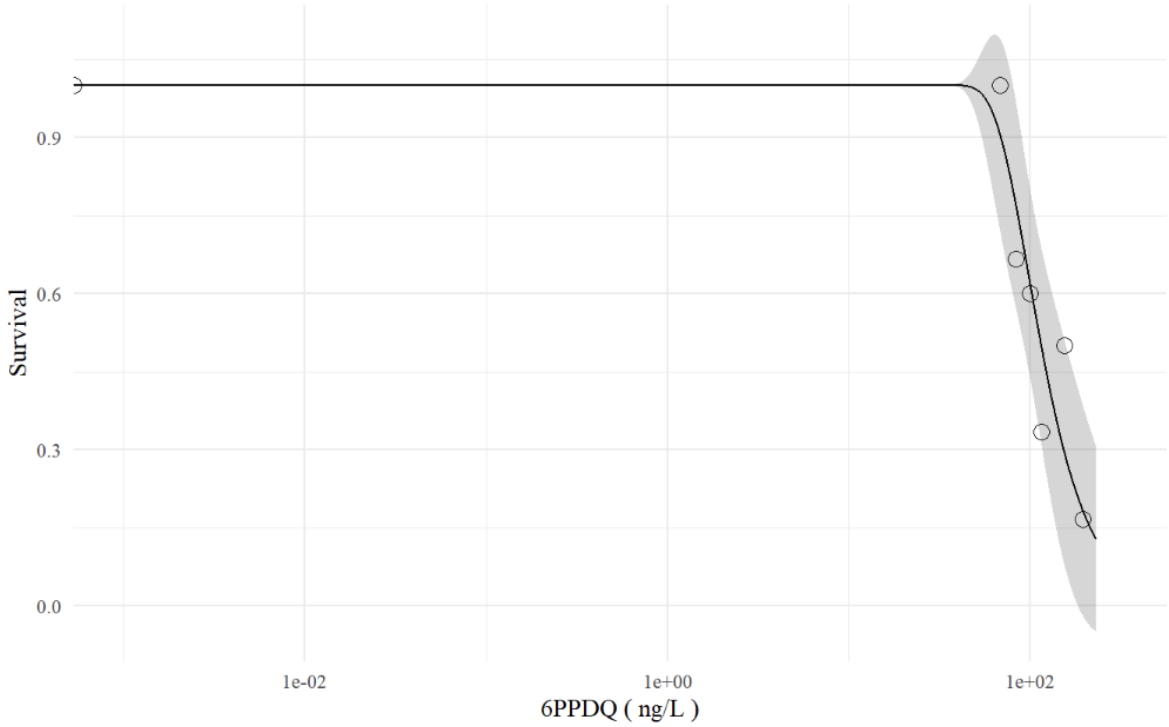
Publication: Tian et al. 2022
Species: *Oncorhynchus kisutch*
EPA-Calculated LC_{50S}: 0.09693 and 0.1152 µg/L (or 0.07752 and 0.09216 after adjusting for loss)
Concentration-Response Model Estimates:

Parameter	Estimate	Std. Error	t-stat	p-value
b	-2.536	0.7688	-3.299	0.000972
e	83.88	9.751	8.602	$< 2.2e^{-16}$

Parameter	Estimate	Std. Error	t-stat	p-value
b	-2.338	0.8649	-2.704	0.006854
e	98.51	10.62	9.280	$< 2.2e^{-16}$

Concentration-Response Model Fit:



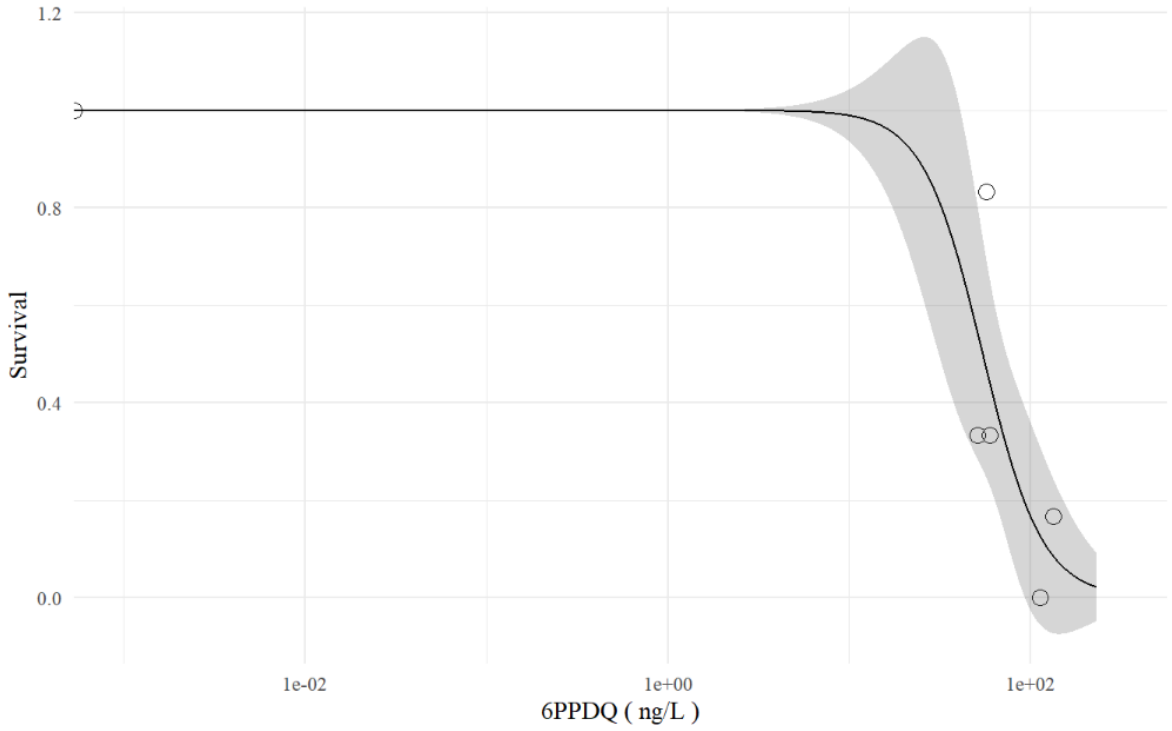


A.2.1.3 Greer et al. 2023a Concentration Response Curve – Oncorhynchus kisutch (Coho salmon)

Publication: Greer et al. 2023a
Species: *Oncorhynchus kisutch*
EPA-Calculated LC_{50}: 0.0546 µg/L
Concentration-Response Model Estimates:

Parameter	Estimate	Std. Error	t-stat	p-value
b	2.659	1.331	1.998	0.04571
e	54.57	9.956	5.481	$4.221e^{-8}$

Concentration-Response Model Fit:



A.2.2 Second most acutely sensitive genus: *Salvelinus* (Salmonid)

Brinkmann et al. (2022) investigated the acute toxicity of N-(1,3-Dimethylbutyl)-N′-phenyl-p-phenylenediamine-quinone (6PPD-q, CAS# 2754428-18-5) to brook trout, *Salvelinus fontinalis* in a static, measured exposure. Chemical purity was not reported in the paper, but a purity of 97% and the name of the manufacturer (Toronto Research Chemicals) was provided by the study authors via subsequent personal communication with the EPA. Stock solutions were prepared using dimethyl sulfoxide (DMSO) to achieve a final solvent concentration of 0.01% (v/v) during exposures. Juvenile brook trout (~1 year old, 17.1 cm, 52.8 g) were obtained from Allison Creek Trout Hatchery (Coleman, AB) and were acclimated at the Aquatic Research Facility (University of Lethbridge) in 150 L inert glass fiber Krescel tanks (four fish per tank, 30% daily water renewal) for 2 weeks prior to exposures. Fish were fed a commercial salmonid feed at a daily rate of 1% of body weight during acclimation. Brook trout were statically exposed

to solvent controls and five measured concentrations of 6PPD-q (0.11, 0.72, 1.35, 2.21, and 4.35 µg/L) for 24 hours in tanks similar to those they were acclimated in (two replicate tanks with four fish each for 6PPD-q treatments and four replicates with four fish each for solvent controls; 56 fish total). The resulting biomass loading rate from this experimental design was 1.41 g/L, which is almost 2 times higher than the EPA's 850 Ecological Effects Test Guidelines recommended rate of 0.8 g/L (U.S.EPA 2016b). Test solutions were continuously aerated, recirculated, and temperature controlled. Solvent control tanks were dosed with the DMSO solvent vehicle at the same level as all other tanks [0.01% (v/v)]. Fish were not fed during testing. Average reported water quality conditions consisted of water temperature of 10.3 ± 0.7°C, pH of 6.74 ± 0.13, dissolved oxygen of 99.8 ± 11.5% and total hardness of 131 ± 2.33 mg/L. Water samples were collected for analytical confirmation of 6PPD-q concentrations ~1 hour after the initial dosing of tanks. Average concentrations of 6PPD-q measured over the exposure periods deviated < 16% from nominal values for all but the low-treatment group (32%). Fish were observed frequently during exposure and were immediately removed once they became moribund. No control mortality was observed. However, there was 100% mortality in the high treatment group within 3 hours of exposure. The author-reported 24-hour LC_{50} was 0.59 µg 6PPD-q/L. The EPA was unable to curve fit the data based on the level of detail provided in the paper and there is no change in the value since the author-reported value was based on average concentrations over the experimental duration. Consistent with the study review approach described in Section 2.2.2 above, this test was classified as quantitative use despite the short duration (24 hours as opposed to 96 hours), in spite of the high biomass loading rate (since the test organisms did not appear to be stressed from the loading rate used and D.O. measurements were at acceptable levels).

One of three species evaluated by **Hiki and Yamamoto (2022)** for 6PPD-q toxicity was the whitespotted char, *Salvelinus leucomaenis ssp. pluvius* via a static-renewal, measured exposure. The methodology employed by the authors was essentially the same as used for *Oncorhynchus masou ssp. masou* (Japanese salmon) and *Salvelinus curilus* (Southern Dolly Varden) discussed in Section 4.2 for the studies that were considered for qualitative use. This species is not a resident species in North America, but other species in this genus are resident, commercially, or ecologically important species. The 6PPD-q (CAS# 2754428-18-5, purity > 95%) was purchased from Cambridge Isotope Laboratories and dissolved in acetonitrile. Fish acclimation and testing was conducted at 14ºC under a 12-hour light-dark photoperiod with aeration provided by a Pasteur pipe/air pump. Prior to testing, fish were fed with a commercial fish food three times per week and maintained in dechlorinated tap water at pH of 7.2, hardness of 68 mg/L, and conductivity of 22.3 mS/m). Acute static-renewal measured lethality tests were performed according to the OECD Test Guideline 203 with slight modifications noted below that were inconsequential to the test use in the derivation of this screening value. Seven juvenile fish < 1 year old (weighing an average of 3.2 g/fish and with an average length of 7.0 cm; personal communication with Kyoshiro Hiki, March 2023) were exposed for 96 hours in a 19 cm x 19 cm x 20 cm height glass tank containing approximately 5 L of dechlorinated tap water spiked with five concentrations (0.16, 0.36, 0.82, 1.3, and 3.5 µg/L) of 6PPD-q, measured at the start of the experiment. There was one exposure chamber for each treatment group. Although two or more replicate exposure chambers per treatment are preferred, one chamber is acceptable within the EPA's 850 Ecological Effects Test Guidelines. This experimental design resulted in a biomass loading rate of 4.48 g/L, which is over 5 times higher than the EPA's 850 Ecological Effects Test Guidelines recommended rate of 0.8 g/L (U.S.EPA 2016b). The carrier solvent control was

dosed with acetonitrile at 0.012% (v/v). Average reported water quality consisted of a water temperature of 14.4 ± 0.3 ºC, dissolved oxygen > 60% saturation, and pH 7.5 ± 0.3, respectively. Fish were not fed during the test. Every 24 hours surviving fish were transferred to a glass tank containing 5 L of a newly-prepared test solution to keep exposure concentrations stable. The measured 6PPD-q concentrations were 54 – 109% of the nominal concentrations just after water renewal. Concentrations of 6PPD-q were also measured at the start and end of each test solution renewal period and it was determined that 47 – 97% of the detected 6PPD-q was lost within the first 24 hours of water renewal. All observed lethality occurred within 24 hours of exposure at all concentrations and there was no solvent control mortality. The author-reported 96-hour LC_{50} was 0.80 µg 6PPD-q/L, based on the measured concentrations at the start of the water renewal; the author-reported 96-hour LC_{50} was 0.51 µg /L 6PPD-q based on time weighted average concentrations. The EPA curve fit the C-R data to calculate an LC_{50} value based on time weighted average concentrations. This study was deemed acceptable for quantitative use despite the high biomass loading as there was 0% mortality in the controls and D.O. saturation remained > 60%, indicating that the test organisms were not stressed by this biomass loading rate. The EPA-calculated LC_{50} was 0.5709 µg/L 6PPD-q, which was acceptable for quantitative use.

Roberts et al. (2024) examined the effects of 6PPD-q (97% purity, purchased from Toronto Research Chemicals) on lake trout (*Salvelinus namaycush*) in a 96-hour acute exposure. Eggs sourced from ten field collected females were fertilized with eggs from two males and maintained in the dark at 10 ± 0.5°C in a flow-through heath tray system at the University of Saskatchewan, Aquatic Toxicology Research Facility until they reached the eyed stage. Water temperature, dissolved oxygen (D.O.), ammonia, and hardness were checked twice a day, and any dead embryos were removed. Each test chamber (2.5 L tanks made of an unspecified

material) received one of four nominal concentrations of 6PPD-q (0.1, 0.3, 0.9, and 2.7 µg/L) or a control, for a total of 25 tanks. All test concentrations and controls included 0.01% DMSO as a solvent. Exposure water was sampled in each tank at the beginning of the experiment, after 24 hours and three additional times during the experiment. For each individual water sample, 950 µL of exposure water was spiked with 50 µL of deuterium-labeled standard solution (1 mg/L 6PPD-q) and frozen at -20°C in amber autosampler vials until analysis. Water samples were analyzed using ultra-high-performance liquid chromatography in tandem with high-resolution mass spectrometry (LC-MS/MS; Challis et al. 2021). Time weighted average 6PPD-q concentrations during the 96-hour exposure period were (<0.10, 0.16, 0.55, and 2.1 µg/L). A concentration of 0.05 µg/L, or half of the detection limit, was used to represent the lowest treatment level when calculating the acute LC_{50}. No 6PPD-q was reported in the solvent controls. Fifteen eight-week-old post-swim up juvenile lake trout were randomly added to each tank. Tanks were maintained at 10 ± 0.5°C, and each tank received a daily 70% water change. Water quality during the acute test was not reported. However, ammonia, nitrite, nitrate, hardness, pH, and D.O. were measured weekly during a 45-day chronic study with a similar test design, and concentrations were determined to be acceptable. Test organisms were not fed during the acute study. Fish were monitored for behavioral changes and mortality every hour for the first seven hours, and then twice a day for the remainder of the test. Acute mortality was observed at the highest test concentration within the first hour and continued throughout the test. Behavioral changes were observed at the 0.55 and 2.1 µg/L test concentrations throughout the study, including loss of coordination, gasping, and surface swimming. All fish that exhibited these behaviors died within six hours. No fish in the control or two lowest treatment concentration chambers exhibited any behavioral changes, and control survival was 100%. The author-

calculated LC_{50} was 0.50 µg/L (based on time weighted average concentrations). The EPA re-calculated the LC_{50} from C-R data based on time weighted average concentrations. The EPA-calculated LC_{50} was 0.5186 µg/L 6PPD-q, which was acceptable for quantitative use.

A.2.2.1 Brinkmann et al. 2022 Concentration Response Curve – Salvelinus fontinalis (Brook trout)

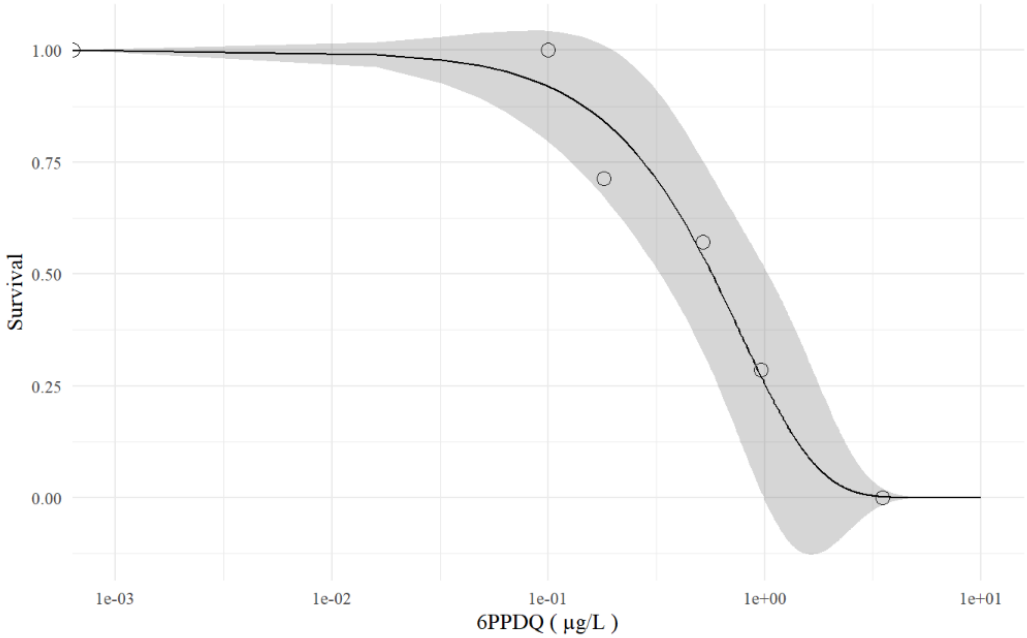
Publication: Brinkmann et al. 2022
Species: *Salvelinus fontinalis*
EPA-Calculated LC_{50}: Not calculable, concentration-response data not available

A.2.2.2 Hiki and Yamamoto 2022 Concentration Response Curve – Salvelinus leucomaenis ssp. pluvius (Whitespotted char)

Publication: Hiki and Yamamoto 2022
Species: *Salvelinus leucomaenis*
EPA-Calculated LC_{50}: 0.5709 µg/L
Concentration-Response Model Estimates:

Parameter	Estimate	Std. Error	t-stat	p-value
b	1.209	0.4135	2.923	0.0035
e	0.7731	0.2137	3.618	0.0002967

Concentration-Response Model Fit:

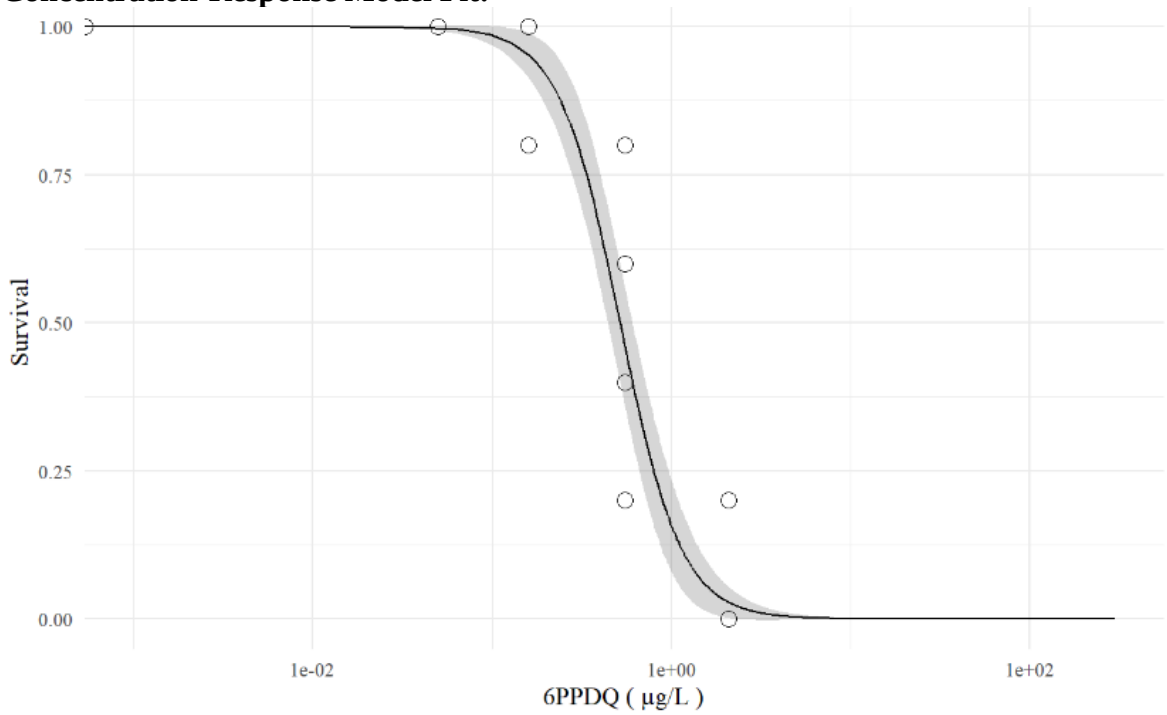


A.2.2.3 Roberts et al. 2024 Concentration Response Curve – Salvelinus namaycush (Lake trout)

Publication: Roberts et al. 2024
Species: *Salvelinus namaycush*
EPA-Calculated LC_{50}: 0.5186 µg/L
Concentration-Response Model Estimates:

Parameter	Estimate	Std. Error	t-stat	p-value
b	2.539	0.3187	7.968	$1.581e^{-15}$
e	0.5186	0.04145	12.51	$< 2.2e^{-16}$

Concentration-Response Model Fit:



A.2.3 Third most acutely sensitive taxon: *Oncorhynchus mykiss* (rainbow trout - salmonid)

Brinkmann et al. (2022) performed a 96-hour static-renewal, measured acute test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q) with the rainbow trout, *Oncorhynchus mykiss*. Rainbow trout used for testing were obtained from Lyndon Hatcheries, New Dundee, ON, and cultured in-house under flow through conditions in University of Saskatchewan testing facility water until testing age (~2-year-old juveniles). Chemical purity was not reported in the paper, but a purity of 97% and the name of the manufacturer (Toronto Research Chemicals) was provided by the study authors via subsequent personal communication with the EPA. A stock solution was prepared by dissolving 6PPD-q in a dimethyl sulfoxide (DMSO) solution (0.01% v/v). Test chambers were 700 L glass-fiber Min-o-Cool tanks with 500 L of test solution per tank. Each tank contained five fish (weighing an average of 97.5 g), with two replicate tanks per treatment and a control that was replicated three times. The resulting biomass loading rate averaged 0.975 g/L, which is slightly higher than the 0.8 g/L recommended

in the EPA's 850 Ecological Effects Test Guidelines (U.S.EPA 2016b). Stock solution was added to tanks to make five treatment concentrations (0.09, 0.72, 1.38, 2.78 and 5.33 µg/L; average measured 6PPD-q) plus a solvent control. Average measured test concentrations were based on the quantification of 6PPD-q in samples of test solution collected before and after each renewal. With the exception of the lowest test concentration, measured concentrations were within ± 12% of nominal. The lowest concentration was within 27% of the nominal concentration. Measured concentrations decreased between daily (every 24 hours) solution renewals by an average 44.2% at the lowest concentration and 26.5% and 26% at the two next higher concentrations. All tanks, including solvent controls, had DMSO solvent concentrations of 0.01% v/v. Test water was continuously aerated and circulated, and each tank underwent a daily water exchange of 75%. Fish were not fed during testing. Water quality conditions throughout the experiment consisted of a water temperature of 12.8 ± 0.8℃, pH of 8.35 ± 0.45, oxygen saturation of 92.8 ± 13.2%, ammonia 0.14 ± 0.15 mg/L, and hardness 132 ± 6.8 mg/L. No solvent control mortality was observed. The authors noted that for rainbow trout, the first signs of morbidity did not manifest until 7 hours after commencing exposures and maximum mortalities occurred at 60 hours. The author-reported 96-hour LC_{50} was 1.00 µg 6PPD-q/L based on average concentrations over the experimental duration. The EPA was unable to curve fit the C-R data based on the level of detail provided in the paper and therefore the author-reported LC_{50} was used. This test was determined to be acceptable for quantitative use despite the slightly elevated biomass loading rate compared to the EPA's 850 Ecological Effects Test Guidelines since there was no control mortality and oxygen saturation was 92.8%.

Di et al. (2022) performed a 96-hour static-renewal, measured acute test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, purity ≥98%, obtained from

Hengyang Jiaxuan Biotechnology Co., Ltd.) with the rainbow trout, *Oncorhynchus mykiss*. Juvenile rainbow trout (3.0 ± 0.3 g) used for testing were obtained from a fish market in Hangzhou, China, and were acclimated to facility water for two weeks prior to testing. An initial stock solution (1,000 mg/L) was made by dissolving 6PPD-q in acetonitrile (0.05% v/v). Stock solution was added to test chambers containing 24 L of dechlorinated water to make five treatment concentrations (1.75, 1.92, 2.12, 2.33, and 2.56 µg/L of measured 6PPD-q) plus a solvent control. There were three tanks per treatment (and control). Each tank contained eight fish, resulting in an average biomass loading rate of 1 g/L. This biomass loading rate was slightly higher than that recommended in the EPA's 850 Ecological Effects Test Guidelines of 0.8 g/L (U.S.EPA 2016b). Solvent control tanks included acetonitrile (solvent amount by volume not provided) added to dechlorinated tap water. A 50% water exchange was conducted after 48 hours. The study authors did not report a comparison of measured concentrations to nominal in order to track potential fluctuations of 6PPD-q over the exposure duration. Water temperature averaged 16 ± 1°C, and the test was conducted using a daily cycle of 14 hours light, 10 hours dark. Fish were not fed during the test, and dead fish were removed when first observed. The author-reported 96-hour LC_{50} was 2.26 µg/L 6PPD-q. The EPA re-calculated the LC_{50} (2.232 µg/L) from C-R data based on measured concentrations provided by study authors in the paper, but it is unclear when these measurements were taken during the exposure. It was assumed that reported measured concentrations were initial concentrations; therefore, the EPA-calculated LC_{50} value was adjusted to lower the value by 20% in order to account for loss of 6PPD-q over the experiment. The adjusted EPA-calculated LC_{50} was 1.786 µg/L 6PPD-q. This study was determined to be acceptable for quantitative use despite the slightly elevated biomass loading

rate since the test organisms did not appear to be stressed (0% control mortality, D.O. saturation > 60%, ammonia not reported).

Nair et al. (2023) conducted a 96-hour measured, static acute test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q) with the rainbow trout, *Oncorhynchus mykiss*. Chemical purity was not given in the paper; however, through personal communication, the study authors provided the EPA with a purity of 95%, and indicated the chemical was synthesized in the laboratory. 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- 0.7 g), 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 five nominal concentrations (0.2, 0.8, 3, 12 and 25 µg/L) of 6PPD-q by spiking ~2 mL of methanol stock solution into 20 L of water, with measured concentrations of less than detection limit, 0.29, 1.94, 11.2 and 40.0 µg/L 6PPD-q, respectively. Three replicates were tested for each treatment group, with 10 fish 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 under 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). All fish exposed to the nominal 0.8 µg/L of 6PPD-q survived the first

day of exposure, but mortality increased from 30% at Day 2, to 43% at Day 4. The author-reported 96-hour LC_{50} was 0.64 µg 6PPD-q/L. The EPA-calculated LC_{50} (0.3701 µg/L) was based on measured concentrations provided by study authors in the paper, but it is unclear when these measurements were taken in the exposure duration. It was assumed to be initial measured concentrations; therefore, the EPA-calculated LC_{50} value was adjusted to lower the value by 20% in order to account for loss of 6PPD-q over experiment duration. The adjusted EPA-calculated LC_{50} was 0.2961 µg 6PPD-q/L, which was acceptable for quantitative use.

A.2.3.1 Brinkmann et al. 2022 Concentration Response Curve – Oncorhynchus mykiss (Rainbow trout)

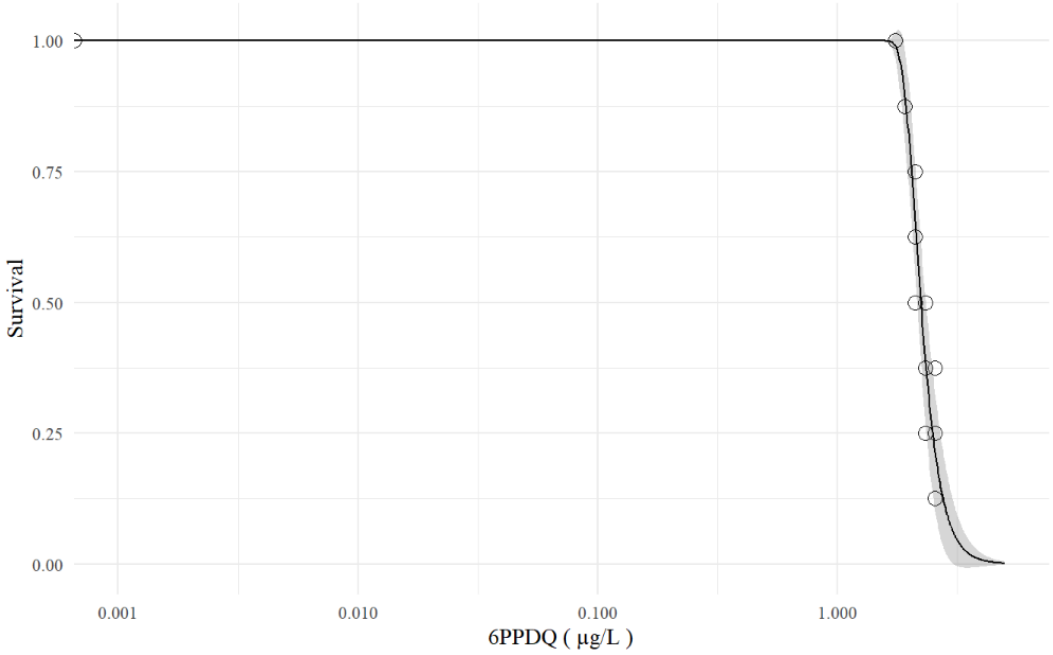
Publication: Brinkmann et al. 2022
Species: *Oncorhynchus mykiss*
EPA-Calculated LC_{50}: Not calculable, concentration-response data not available

A.2.3.2 Di et al. 2022 Concentration Response Curve – Oncorhynchus mykiss (Rainbow trout)

Publication: Di et al. 2022
Species: *Oncorhynchus mykiss*
EPA-Calculated LC_{50}: 2.232 µg/L (or 1.786 µg/L after adjusting for loss)
Concentration-Response Model Estimates:

Parameter	Estimate	Std. Error	t-stat	p-value
b	-7.713	1.335	-5.776	$7.64e^{-9}$
e	2.128	0.040	53.07	$< 2.2e^{-16}$

Concentration-Response Model Fit:

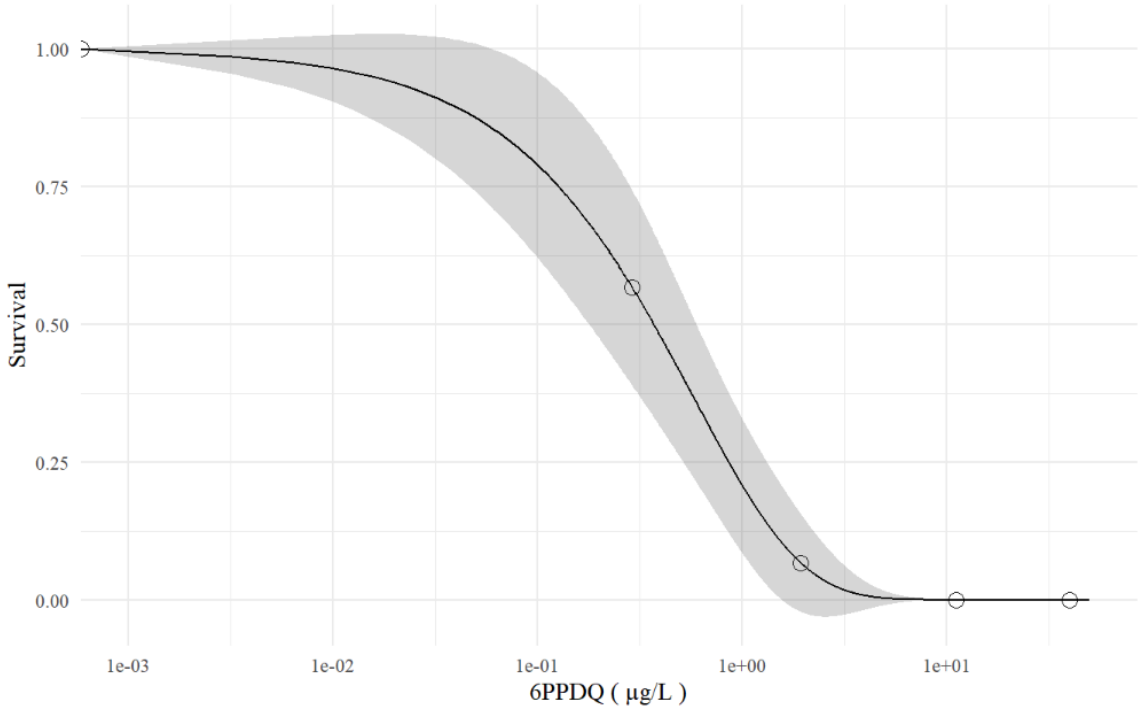


A.2.3.3 Nair et al. 2023 Concentration Response Curve – Oncorhynchus mykiss (Rainbow trout)

Publication: Nair et al. 2023
Species: *Oncorhynchus mykiss*
EPA-Calculated LC_{50}: 0.3701 µg/L (or 0.2961 µg/L after adjusting for loss)
Concentration-Response Model Estimates:

Parameter	Estimate	Std. Error	t-stat	p-value
b	0.822	0.1977	4.157	$3.22e^{-5}$
e	0.5782	0.1415	4.085	$4.402e^{-5}$

Concentration-Response Model Fit:



A.2.4 Fourth most acutely sensitive genus: *Pimephales* (minnow)

Anderson-Bain et al. (2023) performed a 96-hour static-renewal, measured acute test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, purity not reported in paper) with the fathead minnow, *Pimephales promelas*. Chemical purity was not reported in the paper, but a purity of 97% and the name of the manufacturer (Toronto Research Chemicals) was provided by the study authors via subsequent personal communication with the EPA. Sexually mature fathead minnows were from a brood stock maintained in the Aquatic Research Facility at the University of Lethbridge (original source: Aquatic Research Organisms Inc., NH). The culture was maintained at 22 ℃, a 14:10 hour light: dark photoperiod, and fed a diet of Sally's bloodworms (San Francisco Bay Brand®, San Francisco, CA), hatched brine shrimp (*Artemia salina*, Brine Shrimp Direct, Ogden, UT) and ground Tetramin (Tetra®, Blacksburg, VA), *ad libitum*. Water quality parameters (pH, dissolved oxygen, ionized and unionized ammonia,

nitrate and nitrite) were tested daily. Because exposure to 20 µg/L of 6PPD-q in the range-finding test did not cause lethality, sublethal effects of acute exposure were assessed in a 96-hour exposure that followed recommendations of OECD Test No. 208. Briefly, fathead minnows (adult; 3 males and 3 females per tank) were acclimated for four days in 45 L glass aquaria at 22 °C with an 80% daily water renewal. Tanks were fitted with lids and continuous aeration was supplied. Fish were fed ground Tetramin once daily for the first 3 days of acclimation, and food was withheld on the final day. Following acclimation, fathead minnows were exposed for 96 hours in duplicate (2 tanks per treatment, 6 fish per tank) to 6PPD-q at nominal concentrations of 0, 0.2, 2, or 20 µg/L. A 50% water renewal was conducted daily to replenish concentrations of 6PPD-q. A 990 µL sample of water was taken before and 1 hour after addition of 6PPD-q, and immediately before and after each water change. Water samples were taken from control tanks at the same time points. Samples were stored at -20 °C until they were analyzed. The time-weighted average concentrations of 6PPD-q measured over the 96-hour exposure were 0.09, 0.85, and 9.65 µg/L, which were approximately 44, 43 and 48 percent of nominal values in the low, medium, and high treatment groups, respectively. Fish were fed ground Tetramin® at 48 hours of exposure. The final concentration of dimethyl sulfoxide (DMSO) in all tanks was 0.01% (v/v). Exposures included both water only and solvent controls (0.05% DMSO). Water quality parameters (pH, dissolved oxygen, ionized and unionized ammonia, nitrate and nitrite) were tested. At 96 hours of exposure, fish were removed from tanks and immediately euthanized in buffered tricaine methanesulfonate (MS-222, Millipore-Sigma). Mass (g) and fork length (mm) were recorded for each fish to determine condition factor (K). Exposure to 6PPD-q for 96 hours did not cause mortality of adult fathead minnows. There were also no effects on body length, body mass, or K. The author-reported 96-hour LC_{50} was >9.65 µg 6PPD-q/L, based on average

concentrations over the experimental duration used. The EPA was unable to curve fit the C-R data based on the level of detail and lack of effects provided in the paper. The author-reported LC_{50} was acceptable for quantitative use.

A.2.4.1 Anderson-Bain et al. 2023 Concentration Response Curve – Pimephales promelas (Fathead minnow)

Publication: Anderson-Bain et al. 2023
Species: *Pimephales promelas*
Genus: *Pimephales*
EPA-Calculated LC_{50}: Not calculable, concentration-response data not available

A.2.5 Fifth most acutely sensitive genus: *Planorbella* (snail)

Prosser et al. (2023) conducted a 24-hour static, measured test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, >99.8% purity, purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada)) with the file ramshorn snail, *Planorbella pilsbryi*. Test methodology followed that outlined in (Osborne et al. 2023). Adult *P. pilsbryi* snails with shell lengths of 13-16 mm were taken from a continuous culture at the University of Guelph and placed into Pyrex dishes filled with dechlorinated City of Guelph tap water and lined with plastic sheets. The adult snails were then allowed to lay egg masses over a 12-hour period. After the laying period, the adult snails were returned to the culture aquaria and egg masses were placed in 18 wells of a 24-well plate. Negative controls, solvent controls (0.02% solvent by volume), and 3.75, 7.5, 15.0, and 30.0 µg/L treatments were prepared (n = 3). Each exposure solution was sub-sampled when prepared and stored at -20 °C for analysis to confirm the concentration of 6PPD-q (quantified using solid phase extraction followed by ultraperformance liquid chromatography (UPLC) and tandem mass spectrometry (MS-MS). The snail eggs were exposed to their respective exposure solution for 24 hours in the wells, then the exposure solutions were replaced by clean water. This was done to simulate a 24-hour exposure

to 6PPD-q. A photo of each egg mass was taken every 48 hours throughout the duration of the test (10 days). The water in the wells was also replaced every 48 hours throughout the remainder of the test. The well plate was placed into a controlled environmental chamber maintained at 24-25°C throughout the entire test. Endpoints taken for this experiment were percent mortality and number of snails that hatched. Exposure solutions were sampled for 6PPD-q analysis when the solutions were prepared on day 0. Water chemistry measurements of pH, dissolved oxygen, ammonia and temperature were taken at the initiation and completion of the experiment and ranged from 7.1-7.6 SU, 7.86-8.22 mg/L, 0.0 mg/L and 24-25 °C, respectively. The measured concentration of 6PPD-q was consistently lower than the nominal concentration. The percentage difference between measured and nominal concentration at day 0 ranged from 25.3% to 45.3%. Control organism hatch was 100% at test termination. The author-reported 24-hour LC_{50} (followed by nine days in clean water) was >11.7 µg/L 6PPD-q. The highest test concentration also had no significant adverse effect on snail hatching. The EPA was unable to curve fit the C-R data based on the level of detail provided in the paper and lack of effects. The author-reported value (>11.7) was based on average concentrations over the experimental duration used and was acceptable for quantitative use.

A.2.6 Sixth most acutely sensitive taxon: *Oncorhynchus nerka* (sockeye salmon)

In addition to coho and chinook salmon, **Greer et al. (2023a)** also conducted a 24-hour unmeasured, flow-through acute test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, 97.5% purity, obtained from HPC Standards, Atlanta, GA) with sockeye salmon, *Oncorhynchus nerka*. Juvenile sockeye salmon (mean age of 625 days old with an average body weight of 6.46 g wet weight) used for testing were obtained from the Redfish Lake population through the NOAA Northwest Fisheries Science Center, Manchester Research Station (WA). Fish were housed in 175 L flow-through tanks supplied with freshwater from Lake

Washington, UV-treated and filtered at the US Geological Survey Western Fisheries Research Center in Seattle, WA (reported process or dilution water). Water temperature was maintained at 10°C. Fish were fed ~ 2% of body weight every other day with commercial pellets. 6PPD-q was dissolved in dimethyl sulfoxide (DMSO) to make a concentrated stock solution. The stock solution was further diluted in process water for the toxicity test. Sockeye salmon (n= 4/tank) were exposed to 6PPD-q in 9 L tanks containing 8 L of water supplied with constant aeration via air stone. Tanks were set inside a 275 L flow-through circular tank with a low flow of temperature-controlled dilution water to maintain water temperature (10°C). Individuals were allowed to acclimate for a minimum of 1 h prior to exposure. Fish were not fed for 24 h prior to exposure. To ensure thorough and rapid mixing during exposures, 2 L of fresh dilution water were vigorously poured into each tank while 1 L of the appropriate 6PPD-q stock solution was added.

Fish were exposed to 6PPD-q concentrations ranging up to 50 µg/L (n = 9 exposures). Each concentration was typically administered to one tank per day (2-3 concentrations, one tank each). Replication was done across different days, and the number of replicates varied across concentrations. Unlike with coho and chinook salmon, no mortality was observed in sockeye salmon at nominal concentrations up to 50 µg/L, and thus, lethal concentrations could not be calculated. No mortality was also observed in any solvent control treatments (amount of DMSO not provided). Therefore, the estimated 24-hour author-reported LC_{50} for 625-day old sockeye salmon was >50,000 ng 6PPD-q/L, or >50 µg/L. The EPA did not curve fit anything outside the most sensitive species and author-reported values were used with the exception of adjustments for loss of 6PPD-q over the exposure duration. The author reported values are based on nominal concentrations since this test was unmeasured. The LC_{50} value was adjusted to lower the value

by 20% in order to account for loss of 6PPD-q over the experiment. The LC_{50} adjusted for loss is equal to >40 µg/L, which was acceptable for quantitative use.

A.2.7 Seventh most acutely sensitive taxon: *Oncorhynchus tshawytscha* (chinook salmon)

Lo et al. (2023) also conducted a 24-hour static, measured acute test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, 97.26% purity, obtained from HPC Standards, Atlanta, CA) with chinook salmon (*Oncorhynchus tshawytscha*). This second test was similar to that summarized above for coho salmon (see also Section 3.1.1.1). Juvenile chinook salmon (3 weeks post-swim up with an average body weight of 0.829 g wet weight) used for testing were obtained from Chehalis River Hatchery (Agassiz, BC, Canada). After feeding for one week, fish were transported to the Pacific Science Enterprise Centre (PSCE) and then reared for two weeks in 125 L flow-through glass-fiber tanks prior to 6PPD-q exposure. Rearing conditions were consistent with Environment Climate Change Canada's *Biological Test Method: Acute Lethality Using Rainbow Trout* (ECCC 1990). Well water was used for culturing and exposures. Stock solutions of 6PPD-q were prepared using absolute ethanol, resulting in a final solvent concentration of 0.01% (v/v). These stock solutions were prepared 24 hours before exposure. For the coho salmon exposures, a single stock solution was created, and subsequent stock solutions were made by serial dilution. Test chambers were 20 L glass tanks with 18 L of test solution and 11 fish per replicate tank, which resulted in a biomass loading rate of 0.507 g/L. The 6PPD-q exposures consisted of five nominal concentrations of 5.72, 10.29, 18.52, 33.33, 60.00 µg 6PPD-q/L, corresponding to measured concentrations of 3.112, 5.266, 11.32, 25.81, and 67.31 µg/L, respectively, along with separate well water and solvent (0.01% ethanol) controls, with four replicates each. All exposures were conducted under static conditions with continuous aeration, a photoperiod of 16:8 (light:dark) and light intensity of 100 – 500 lux. Fish were not fed during testing. Average water quality conditions based on measurements taken at

the initiation and termination of the test consisted of a water temperature of 13.6 ± 0.3, dissolved oxygen (D.O.) > 86%, pH between 6.7 – 7.0, and conductivity of 89.8 mg/L. Test concentrations of 6PPD-q were measured in two replicates from each of the treatment groups at the initiation and termination of the test (the same replicates at each sampling event). Average concentrations of 6PPD-q at the test initiation deviated 22.8 ± 16.9% (3.3 – 49.3%) from the nominal concentration. The average loss of 6PPD-q after 24 hours of exposure was 41.0 ± 10.8%, as reported by the study authors. Survival in the well water and solvent controls were 100%. The study authors reported that the average survival rate of chinook salmon in the highest treatment group (67.31 µg 6PPD-q/L) was 61.4%. Toxicity symptoms (e.g., gasping, loss of equilibrium, erratic swimming) were observed in the highest test concentration during the fifth hour of exposure. The author-reported 24-hour LC_{50} was > 67.31 µg 6PPD-q/L. The EPA did not curve fit anything outside the most sensitive species and author-reported values were used with the exception of adjustments for loss of 6PPD-q over the exposure duration. The author-reported value was assumed to be initial concentrations and therefore the LC_{50} value was adjusted to lower the value by 20% in order to account for loss of 6PPD-q over experiment duration. The EPA adjusted LC_{50} was >53.85 µg 6PPD-q/L, which was acceptable for quantitative use as a high greater than value, compared to the other test data used.

Greer et al. (2023a) also conducted a 24-hour partially-measured, flow-through acute test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, 97.5% purity, obtained from HPC Standards, Atlanta, GA) with chinook salmon, *Oncorhynchus tshawytscha*. Juvenile chinook salmon (mean age of 582 days old with an average body weight of 12.1 g wet weight) used for testing were obtained from Dworshak populations at the US Fish and Wildlife Service, Dworshak National Fish Hatchery (ID). Fish were housed in 175 L flow-through tanks

supplied with freshwater from Lake Washington, UV-treated and filtered at the US Geological Survey Western Fisheries Research Center in Seattle, WA (reported process or dilution water). Water temperature was maintained at 10°C. Fish were fed ~ 2% of body weight every other day with commercial pellets.

6PPD-q was dissolved in dimethyl sulfoxide (DMSO) to make a concentrated stock solution. The stock solution was diluted in process water for the toxicity test. Chinook salmon (n= 6/tank) were exposed to 6PPD-q in 38 L tanks containing 30 L of water supplied with constant aeration via air stone. Tanks were set inside a 275 L flow-through circular tank with a low flow of temperature-controlled dilution water to maintain water temperature (10°C). Individuals were allowed to acclimate for a minimum of 1 h prior to exposure. Fish were not fed for 24 hours prior to exposure. To ensure thorough and rapid mixing during exposures, 2 L of fresh dilution water were vigorously poured into each tank while 1 L of the appropriate 6PPD-q stock solution was added.

Fish were exposed to 6PPD-q concentrations ranging up to 50 µg/L (n = 11 exposures). Each concentration was typically administered to one tank per day (2-3 concentrations, one tank each). Repeated exposures were conducted across different days, and the number of exposures varied by day and across concentrations. The approach was taken to focus on concentrations defining the slope of the curve. Both nominal and measured concentrations were used to fit the curve for the chinook LC_{50}.

Mortalities were monitored for 24 hours. Mass and fork length were measured for each individual. No mortality was observed in any solvent control treatments (amount of DMSO not provided). Water samples were collected from each tank within the first 30 seconds of exposure. Select samples were analyzed at the U.S. Geological Survey, Organic Geochemistry Research

Laboratory by a direct-inject Waters Corporation Acquity H-Class Bio UPLC/Sciex API 5500 triple quadrupole mass spectrometer (UPLC/MS/MS). Photoperiod and light intensity were not reported, nor were average water quality conditions based on measurements taken during the flow through test. There was no information in the publication about time of death or immobility, but authors noted that all fish exhibited the suite of symptoms associated with URMS. The author-reported 24-hour LC_{50} for 582-day old chinook salmon was 82,100 ng 6PPD-q/L, or 82.1 µg/L. The EPA did not curve fit anything outside the most sensitive species and author-reported values were used with the exception of adjustments for loss of 6PPD-q over the exposure duration. The author-reported LC_{50} value was based on a combination of measured and nominal concentrations. Since this test was not curve fit by the EPA, the LC_{50} value was adjusted to lower the value by 20% in order to account for loss of 6PPD-q over experiment duration. The EPA adjusted LC_{50} was 65.68 µg/L 6PPD-q, which was acceptable for quantitative use.

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

Varshney et al. (2022) performed a 96-hour static-renewal, unmeasured acute test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, > 98.0% purity, obtained from Cymit Quimica Chemical, Barcelona, Spain) with the zebrafish, *Danio rerio*. AB strain embryos (< 16 cell stage) used for testing were obtained 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 (nominal concentrations of 0 - 400 µg 6PPD-q/L). 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). Test solution was added to the well plate 24 hours prior to introducing the embryos. Each well plate was covered and incubated at 26 ± 1°C under a daily cycle of 12 hours light, 12 hours dark.

Test solutions were renewed daily. The study authors did not report a comparison of measured concentrations to nominal in order to track potential fluctuations of 6PPD-q over the exposure duration. Oxygen saturation averaged 87.7% and pH averaged 6.81 throughout the experiment. All control organisms underwent normal development, with zero mortality. Mortality was observed at 24 hours across treatments >100 µg/L 6PPD-q. The author-reported LC_{50} concentrations decreased as exposure length increased: 308.67 µg/L 6PPD-q at 24 hours to 132.9 µg/L 6PPD-q at 96 hours. The 96-hour author-reported LC_{50} was 132.9 µg 6PPD-q/L. The EPA did not curve fit anything outside the most sensitive species and author-reported values were used with the exception of adjustments for loss of 6PPD-q over the exposure duration. The author reported values were based on nominal concentrations since this test was unmeasured, therefore the LC_{50} value was adjusted to lower the value by 20% in order to account for loss of 6PPD-q over the experiment duration. The EPA-adjusted LC_{50} was 106.3 µg/L 6PPD-q, which was acceptable for quantitative use.

A.2.9 Ninth most acutely sensitive genus: *Hexagenia* (mayfly)

Prosser et al. (2023) performed a 96-hour static, measured acute test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, >99.8% purity, purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada)) with the mayfly, *Hexagenia spp*. Mayfly eggs were collected along the shores of Lake St. Clair in Ontario, Canada. When the adult mayflies emerged, eggs were collected from the female mayflies by touching their abdomen to culture water prepared in the laboratory. The culture water was a 50:50 mixture of dechlorinated City of Guelph (Ontario) drinking water and purified water from a Millipore IQ7000® ultrapure water system. The test organisms used in this experiment were a mixture of *Hexagenia rigida* and *Hexagenia limbata*. The eggs were stored at 4 ℃ until used in an experiment. The eggs maintain a viability of >85% over a 1-year period when stored at 4 ℃. In

preparation for testing, eggs were brought up to 22 ±1 °C to hatch. Approximately 400 larvae were added to 25-L glass aquaria which contained a 3 to 4-cm layer of sediment that was collected from Long Point Provincial Park (Long Point, ON, Canada) and sieved to 250 µm and filled with culture water. Twice per week, each aquarium of *Hexagenia* was fed a mixture of 1.876 g of Innovating Science® Cereal Grass Media and 1.250 g of Nutrafin® fish flakes grinded and sieved to 250 µm. The larval mayflies were left to grow in the aquaria until they were used in testing. The test vessel used for acute toxicity testing was a 1-L glass vessel. Each test vessel received 800 mL of their respective exposure solutions and each test vessel was equipped with a disposal glass Pasteur pipette connected to an airline for gentle aeration (~30 bubbles/minute) of the exposure solution. Aeration was used to maintain the dissolved oxygen concentration in the exposure water but was also gentle enough to not generate a current in the test vessel. Three test vessels were prepared as negative controls, i.e., no 6PPD-q present, and three test vessels were prepared as a solvent control (0.02% solvent by volume), as methanol was used in preparation of the exposure solutions. While the experiment was repeated twice with different tests concentrations (author reported as Test A and Test B), only the results from Test B are discussed due to the 12% mortality in the solvent control observed in Test A. Larvae were exposed to nominal concentrations of 10, 100, and 1,000 µg/L (Test B) with five test vessels per exposure concentration. Each exposure solution was sub-sampled for analysis to confirm 6PPD-q concentration, and the solutions were frozen at -20 °C until they could be analyzed. Samples were taken at day 0 for the negative control, solvent control, 10 µg/L, and 1,000 µg/L treatment, and the 10 µg/L treatment was sampled at day 4. Test organisms were sieved from culture aquaria and similar-sized organisms were selected for use in the test. Using a fine paintbrush, five individuals were then placed into 5-mL medicine cups with water and cups were randomly

assigned to test vessels, so each test vessel contained five larval mayflies. A sub-sample of organisms (n = 12) was photographed using a Nikon SMZ-18® microscope and NIS-Elements® software to assess the average length of larvae, which was 13.45 mm. Artificial burrows made of glass tubing were placed in each test vessel. The test vessels were placed in a controlled environmental chamber at 23 ℃ in the dark for 96 hours. At each 24-hour interval, vessels were checked for mortalities. If mortalities had occurred, casualties were removed from their test vessel with sterile forceps. Water chemistry measurements of pH, dissolved oxygen, specific conductivity, ammonia and temperature were taken at the initiation and completion of the experiment and ranged from 6.5-7.5 SU, 5.89-8.10 mg/L, 608-636 µS/cm, 0-0.25 mg/L and 23° C, respectively. The measured concentration of 6PPD-q (quantified using solid phase extraction followed by ultraperformance liquid chromatography (UPLC) and tandem mass spectrometry (MS-MS) was consistently lower than the nominal concentration. The mean measured concentrations were 1.0 µg/L and 232.0 µg/L relative to the nominal concentrations of 10 µg/L, and 1,000 µg/L, respectively. Across all treatments and controls organism mortality was ≤ 8.0%. The author-reported 96-hour LC_{50} was >232.0 µg 6PPD-q/L and was based on average concentrations over the experimental duration used. The EPA was unable to curve fit the data based on the level of detail provided in the paper and lack of effects. Therefore, the author-reported value based on average concentrations was used and was acceptable for quantitative use.

Appendix B Quantitative Acute Estuarine/Marine Toxicity Data

B.1 Summary Table of Acceptable Quantitative Estuarine/Marine Acute 6PPD-q Toxicity Studies

Species (lifestage)	Method[a]	Test Duration	Chemical / Purity	pH	Temp. (°C)	Salinity (ppt)	Effect	Author Reported Effect Conc. (µg/L)	Species Mean Acute Value (µg/L)	Reference
Amphipod (neonate, ≤7d), *Parhyale hawaiensis*	S, U	96 hr	6PPD-q ≥97%	7.95-8.36	24	31.78-32.24	LC50	>500	>500	Botelho et al. (2023)

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

B.2 Detailed 6PPD-q Acute Estuarine/Marine Toxicity Study Summaries and Corresponding Concentration-Response Curves (when calculated for the most sensitive genera)

The purpose of this appendix was to present detailed study summaries for tests that were considered quantitatively acceptable for screening value derivation. A single study summary is presented below reflecting estuarine/marine acute taxonomic sensitivity to 6PPD-q. The EPA did not develop Concentration-response (C-R) models for these acute toxicity values because a screening value could not be derived for estuarine/marine waters given the limited data available.

B.2.1 Most acutely sensitive genus: *Parhyale* (amphipod)

Botelho et al. (2023) performed a 96-hour static, unmeasured acute test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, ≥ 97% purity, purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada)) with the amphipod, *Parhyale hawaiensis*. Neonates (≤ 7 days old) used for testing were obtained from in-house cultures. Organisms were maintained in plastic containers containing 2 L of artificial seawater (Red Sea Salt, salinity 30 ± 2; dissolved oxygen: 6 ± 2 mg/L; pH: 8 ± 1), crushed coral (granulometry #8) as substrate, and at a density of 125 neonates/L. The culture was maintained at 24 ± 2 ℃ under constant aeration and a photoperiod of 12 hour light:dark. Organisms were fed five times per week with commercial food JBL Novo Fect®. Renewal of 50% of the water was performed twice a week to maintain appropriate water quality during culture conditions. Acute toxicity testing using *P. hawaiensis* was performed according to Artal et al. (2018). A stock solution of 6PPD-q was prepared in dimethyl sulfoxide (DMSO) at the solubility limit (5 g/L). 6PPD-q was evaluated in five concentrations ranging from 31.25 to 500 µg/L. Dilutions in the test media contained a maximum concentration of 0.01% DMSO as recommended by OECD (2019). Artificial seawater was used as negative control, and 0.01% DMSO as the solvent control. The test was conducted in 96-well plates and for each concentration. Thirty two neonates were

exposed for 96 hours (1 neonate per well) at 24 ± 2 ℃ and a photoperiod of 12 hour light:dark. Salinity, pH, and dissolved oxygen were measured at the beginning and end of the test and ranged from 31.78-32.24 ppt, 7.95-8.36 SU and 4.06-5.41 mg/L, respectively. At the end of exposure, the number of dead organisms was observed to calculate mortality. Control organism mortality was ≤ 3.1% in the artificial seawater and 0% in the solvent control. Across all 6PPD-q treatments mortality was ≤3.1%. The 96-hour LC_{50} was >500 µg 6PPD-q/L, which was acceptable for quantitative use.

Appendix C Quantitative Chronic Freshwater Toxicity Data

C.1 Summary Table of Acceptable Quantitative Freshwater Chronic 6PPD-q Toxicity Studies

Species (lifestage)	Method[a]	Test Duration	Chemical / Purity	pH	Temp. (°C)	Hardness (mg/L as CaCO3)	Effect	Author Reported Effect Conc. (µg/L)	Species Mean Chronic Value (µg/L)	Reference
Cladoceran (<24 hr), *Daphnia magna*	R, M	21 d	6PPD-q >99.8%	8.4-8.5	20	105.5	NOEC (growth and mortality)	30.2	30.2	Prosser et al. (2023)
Lake trout (alevin), *Salvelinus namaycush*	R, M	45 d	6PPD-q 97%	8.23	10	92.67	LC50	0.39	0.39	Roberts et al. 2024

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

C.2 Detailed 6PPD-q Chronic Freshwater Toxicity Study

The purpose of this appendix was to present 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-q. The EPA did not develop Concentration-response (C-R) models for these chronic toxicity values because a screening value could not be derived for chronic exposures in freshwaters given the limited data available.

C.2.1 Most chronically sensitive genus: *Salvelinus* (trout)

Roberts et al. (2024) examined the effects of 6PPD-q (97% purity, purchased from Toronto Research chemicals) on lake trout (*Salvelinus namaycush*) in a 45-day static-renewal measured chronic exposure. Eggs sourced from ten field collected females were fertilized with eggs from two males and maintained in the dark at 10 ± 0.5°C in a flow-through heath tray system at the University of Saskatchewan, Aquatic Toxicology Research Facility until they reached the eyed stage. Test solution was added to 2.5 L tanks (test chambers), which were individually aerated and allowed to equilibrate for 24-hours prior to test initiation. Each tank received one of six nominal concentrations of 6PPD-q (0.625, 1.25, 2.5, 5, 10, and 20 µg/L) or a control, for a total of 35 tanks. All test concentrations and controls included 0.01% DMSO as a solvent. Exposure water was sampled in each tank 24 hours before the experiment, at the beginning of the experiment, and four separate times during the experiment. For each individual water sample, 950 µL of exposure water was spiked with 50 µL of deuterium-labeled standard solution (1 mg/L 6PPD-q) and frozen at -20°C in amber autosampler vials until analysis. Water samples were analyzed using ultra-high-performance liquid chromatography in tandem with high-resolution mass spectrometry (LC-MS/MS; Challis et al. 2021). Time weighted average 6PPD-q concentrations during the 45-day exposure period were 0.22, 0.58, 1.3, 3.4, and 13.5 µg/L. No 6PPD-q was reported in the solvent controls. Fifteen newly-hatched lake trout alevins

were randomly added to each tank. Tanks were maintained at 10 ± 0.5°C, and each tank received a daily 70% water change. Ammonia (0.03±0.18 ppm), nitrite (0.47±0.58 ppm), nitrate (0.73±0.23 ppm), hardness (92.67±9.27 mg/L), pH (8.23±0.20), and D.O. (93±4.65%) were measured weekly. As test organisms entered the alevin swim-up stage, they were fed brine shrimp (*Artemia sp.*) nauplii once a day, which was increased to twice a day after one week. At the end of the study, surviving fry were euthanized in 150 mg/L buffered tricaine mesylate (MS-222), weighed, and measured for total and standard length. Relatively high mortality was observed during the first four days of the exposure and continued throughout the test. Behavioral changes were not observed; however, deformities were. Deformities occurred as early as three days after testing began, suggesting that 6PPD-q may disrupt growth and development during early life stages. The most common deformity was yolk sac edema, followed by blood pooling and spinal curvature. The highest incidences of yolk sac edema and blood pooling in the caudal fin and eye were at the 3.4 µg/L treatment level, and the highest incidences of spinal curvature were at the 1.3 and 7.6 µg/L treatment level. Author-reported deformities were reported as total counts and were not normalized to mortalities. The authors reported that there were no statistically significant differences in fish length or weight among treatments for surviving fish. Control survival was greater than 80%. The author-reported chronic endpoint was 45-day survival (LC_{50}). The author-calculated LC_{50} of 0.39 µg/L (based on time weighted averages) was considered to be acceptable for quantitative use.

C.2.2 Second most chronically sensitive genus: *Daphnia* (cladoceran)

Prosser et al. (2023) performed a 21-day static-renewal, measured chronic test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, >99.8% purity, purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada)) with the cladoceran, *Daphnia magna*. Organisms used in this study were obtained from a continuous culture that was

maintained at the University of Guelph. The culture was fed following 50% water changes that occurred three times a week. Water changes were performed using culture water which contained 1:1 mixture of dechlorinated City of Guelph and distilled water. A sodium selenate solution with a concentration of 5 g/L was added to the food at 1 mL/L once a week. A 100-mL aliquot of two algal species, *Chlorella vulgaris* and *Raphidocelis subcapitata*, was added to 800 mL culture water to create the water that was used for each water change. The approximate algal cell density in the solution of each species was $2x10^6$ and $3x10^6$ for *C. vulgaris* and *R. subcapitata*, respectively. Cultures were kept under light with an intensity of ~800 lux and a 16:8 h light: dark photoperiod and maintained at an average temperature of 21 ±2 ℃. The chronic toxicity test with *D. magna* was adapted from the Organization of Economic Cooperation and Development (OECD) standardized test method 211 (OECD 2012). To simplify the test, only mortality and growth were assessed. The number of neonates produced was not assessed in this test. Exposure solutions were prepared from a concentrated stock solution of 6PPD-q. The culture water containing *C. vulgaris* and *R. subcapitata* at the densities used in culturing described above was spiked with the stock solution of 6PPD-q to create the different exposure solutions used in the test. Negative control, solvent control (0.02% solvent by volume), 10, 50, and 100 µg/L treatments were prepared. One neonate (<24 hours old) was placed in each test vessel (50-mL glass test tube) and ten replicate test vessels were prepared for each treatment. The size of neonates was measured under the Nikon SMZ-18® microscope. Test vessels were placed in a controlled environmental chamber at 20 ℃ with a 16:8 h light:dark photoperiod. Every two days, test organisms were transferred with a glass Pasteur pipette into test vessels with new exposures solutions. The test was terminated after 21 days, and the size of surviving organisms was measured. The test vessels were observed every 24 hours for mortality. If mortality was

suspected, organisms were placed under a microscope to identify heart movement. If no movement was observed in 15 seconds, the organism was considered dead. At the end of the 21-day exposure, the size of daphnids were measured to assess growth. When new exposure solutions were made on day 0, 8, and 21, they were sampled and analyzed for 6PPD-q before conducting the solution change. Water chemistry measurements of pH, dissolved oxygen, specific conductivity, ammonia and temperature were taken at the initiation and completion of the experiment and ranged from 8.4-8.5 SU, 7.87-8.27 mg/L, 623-644 µS/cm, 0-0.25 mg/L and 20°C, respectively. The measured concentration of 6PPD-q (quantified using solid phase extraction followed by ultraperformance liquid chromatography (UPLC) and tandem mass spectrometry (MS-MS) was consistently lower than the nominal concentration. The percentage difference between measured and nominal concentration at day 0 ranged from 58.0% to 71.0%. Control organism survival was 100% and was ≥90% for all 6PPD-q treatments. The 21-day NOEC for mortality and growth/length was 30.2 µg 6PPD-q/L, which was acceptable for quantitative use. The NOEC, based on the three measured concentrations in the highest test treatment, is most likely lower due to loss of 6PPD-q during testing.

Appendix D Quantitative Chronic Estuarine/Marine Toxicity Data

D.1 Summary Table of Acceptable Quantitative Estuarine/Marine Chronic 6PPD-q Toxicity Studies

Species (lifestage)	Method[a]	Test Duration	Chemical / Purity	pH	Temp. (°C)	Salinity (ppt)	Effect	Author Reported Effect Conc. (µg/L)	Species Mean Chronic Value (µg/L)	Reference
Rotifer, *Brachionus koreanus*	R, U	7 d	6PPD-q Not provided	-	25	30	NOEC (population growth rate)	1,000	1,000	Maji et al. (2023)

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

D.2 Detailed 6PPD-q Chronic Estuarine/Marine Toxicity Study Summaries and Corresponding Concentration-Response Curves (when calculated for the most sensitive genera)

The purpose of this appendix was to present 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-q. The EPA did not develop concentration-response (C-R) models for these chronic toxicity values because a screening value could not be derived for estuarine/marine waters given the limited data available.

D.2.1 Most chronically sensitive genus: *Brachionus* (rotifer)

Maji et al. (2023) conducted a 7-day unmeasured, static-renewal chronic test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, purity not provided, synthesized by ASCA GmbH - Berlin, Germany)) with the marine rotifer, *Brachionus koreanus*. The monogonont rotifer was originally collected at Uljin, South Korea. A single individual was isolated, cloned parthenogenetically, and maintained in the laboratory over successive generations. Rotifers were cultured in artificial seawater (TetraMarine Salt Pro; Tetra, Cincinnati, OH) at 25℃ in 30 practical salinity units (psu) under a 12 hour light:dark photoperiod. Rotifers were fed a live diet of green algae *Tetraselmis sp*. (6 x 10^4 cells/mL). A stock solution was prepared in acetone followed by successive dilutions in artificial seawater at 30 psu. Since acute toxicity was not observed in *B. koreanus* in response to 6PPD-q, chronic toxicity was assessed based on life-cycle parameters such as population growth and fecundity of the rotifer. For chronic experiments, neonate rotifers hatched within 2 hours were used. To collect neonate rotifers, eggs were detached from adult rotifers by vigorous vortexing and collected using a sieve with 120 µm mesh. Neonate rotifers hatched within 2 hours (body size <120 µm) and were then collected from the pool of rotifer eggs. Individual neonates were placed in each well of a six-well culture plate containing 50, 250, 500 or 1,000 µg/L of 6PPD-q. Stock

solutions were made in acetone; details about the use of a solvent or water only controls were not provided. To determine the population growth, the number of rotifers in each well was counted under a stereomicroscope (SZX7, Olympus, Tokyo, Japan) at 24-hour intervals until no further population growth was observed. For fecundity measurements, newborn rotifers were counted using the SZX7 stereomicroscope every 12 hours as an indicator of reproduction until the mature rotifer died. All experiments were conducted in triplicate. During the experiment, rotifers were supplied with a diet of green microalgae (*Tetraselmis sp*.) at a concentration of 6 x 10^4 cells/mL, and approximately 50% of the testing solution containing algae supply was renewed daily to maintain exposure conditions. The 7-day population growth rate NOEC was 1,000 µg 6PPD-q/L, which is acceptable for quantitative use. The accompanying 24-hour LC_{50} of >1,000 µg 6PPD-q/L was classified as qualitative due to lack of exposure details.

Appendix E Acute Qualitative Toxicity Data

E.1 Summary of Freshwater Qualitative Acute Toxicity Data

Species (lifestage)	Method[a]	Test Duration	Chemical / Purity	pH	Temp. (°C)	Hardness (mg/L as CaCO3)	Effect	Reported Effect Concentration (µg/L)	Deficiencies	Reference
Cladoceran (<24 hr), *Daphnia magna*	S, M	48 hr	6PPD-q 83.6%	7.99-8.42	21.6-21.9	-	EC50 (death/ immobility)	>46	Only one exposure concentration	Hiki et al. (2021)
Amphipod (3-5 d), *Hyalella azteca*	R, M	96 hr	6PPD-q 83.6%	8.0	23.5	-	LC50	>43	Only one exposure concentration	Hiki et al. (2021)
White sturgeon (juvenile, ~4.5 yr), *Acipenser transmontanus*	R, M	96 hr	6PPD-q 97%	8.35	12.8	132	LC50	>12.7	Only one exposure concentration; too few organisms per exposure concentration	Brinkmann et al. (2022)
Coho salmon (juvenile, 0-2 yr), *Oncorhynchus kisutch*	S, U	24 hr	6PPD-q ~98%	7.6-7.8	10.0-12	-	LC50	0.79	Duration too short, limited test details	Tian et al. (2021)
Japanese salmon (juvenile, <1 yr), *Oncorhynchus masou ssp. masou*	R, M	96 hr	6PPD-q >95%	7.5	14.4	68	NOEC (mortality)	>3.5	Only one exposure concentration; only four fish per treatment	Hiki and Yamamoto (2022)
Atlantic salmon (alevin), *Salmo salar*	S, M	48 hr	6PPD-q Not reported	6.4	4.7	-	LC50	>5.75	Duration too short	Foldvik et al. (2022)
Brown trout (alevin), *Salmo trutta*	S, M	48 hr	6PPD-q Not reported	6.4	4.7	-	LC50	>8.98	Duration too short	Foldvik et al. (2022)

Species (lifestage)	Method[a]	Test Duration	Chemical / Purity	pH	Temp. (°C)	Hardness (mg/L as CaCO3)	Effect	Reported Effect Concentration (µg/L)	Deficiencies	Reference
Artic char (juvenile, ~3 yr), *Salvelinus alpinus*	R, M	96 hr	6PPD-q 97%	8.35	12.8	132	LC50	>14.2	Only one exposure concentration	Brinkmann et al. (2022)
Southern Dolly Varden (juvenile, <1 yr), *Salvelinus curilus*	R, M	96 hr	6PPD-q >95%	7.5	14.4	68	NOEC (mortality)	>3.8	Only one exposure concentration; only four fish per treatment	Hiki and Yamamoto (2022)
Zebrafish (embryo, <3 hpf), *Danio rerio*	R, M	96 hr	6PPD-q 83.6%	7.7	25.9	-	LC50	>54	Only one exposure concentration	Hiki et al. (2021)
Zebrafish (adult, 4 mo), *Danio rerio*	S, M	12 hr	6PPD-q 98.0%	-	26	-	NOEC (swimming speed and distance)	1,000	Duration too short; atypical endpoint	Ji et al. (2022)
Zebrafish (embryo, 8 hpf), *Danio rerio*	R, U	112 hr	6PPD-q >98%	-	28	-	NOEC (mortality)	1,200	Atypical test duration	Zhang et al. (2023)
Medaka (41 d), *Oryzias latipes*	R, M	96 hr	6PPD-q 83.6%	7.9	24.4	-	LC50	>34	Only one exposure concentration	Hiki et al. (2021)

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

E.1.1 Summary of Acute 6PPD-q Toxicity Studies Considered Qualitatively in the Freshwater Screening Value Derivation

E.1.1.1 Daphnia magna

Hiki et al. (2021) performed a 48-hour static measured test of N-(1,3-dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, 83.6 ± 8.9% purity, synthesized in the laboratory via stepwise Michael addition of aniline and 1,3- dimethylbutylamine to 1,4-benzoquinone) with the cladoceran, *Daphnia magna*. The acute toxicity test followed OECD guideline number 202 (OECD 2004). 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 2004) and an acetone solvent. Treatments included a negative control (details about the use of solvent control were not provided) and a single treatment (46 µg/L measured average concentration). The test was conducted in 50 mL glass beakers, each containing five daphnids, with four replicate beakers per treatment. Dissolved oxygen, pH, temperature, and conductivity ranged from 7.64 - 8.62 mg/L, 7.99 - 8.42, 21.6 - 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 or treatment concentration, resulting in a 48-hour EC_{50} > 46 µg 6PPD-q/L. It was considered acceptable for qualitative use because there was only one test concentration and this test was considered to result in a greater than low value, which typically is not used quantitatively (U.S. EPA 2013).

E.1.1.2 Hyalella azteca

Hiki et al. (2021) also performed a 96-hour static-renewal measured test of N-(1,3-dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, 83.6 ± 8.9% purity, synthesized in the laboratory via stepwise Michael addition of aniline and 1,3-dimethylbutylamine to 1,4-benzoquinone) with the amphipod, *Hyalella azteca*. The acute

toxicity test followed the test method outlined by Environment and Climate Change (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 (details about the use of solvent control were not provided) and a single treatment (43 µg 6PPD-q/L 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, 23.5°C, and 31.0 mS/m, respectively. The photoperiod was 16 hours light and 8 hours dark. No mortality was observed in the control or treatment concentrations. Mortality in the control was 5%, and mortality in the test concentration was 0%, resulting in a 96-hour LC_{50} of > 43 µg 6PPD-q/L. The test was considered acceptable for qualitative use because there was only one test concentration and this test was considered to result in a greater than low value, which typically is not used quantitatively (see Section 2.2.3).

E.1.1.3 Acipenser transmontanus

A third species evaluated by **Brinkmann et al. (2022)** for acute sensitivity to N-(1,3-Dimethylbutyl)-N′-phenyl-p-phenylenediamine-quinone (6PPD-q, CAS# 2754428-18-5) was the white sturgeon, *Acipenser transmontanus* via a static-renewal, measured exposure. Chemical purity was not reported in the paper, but a purity of 97% and the name of the manufacturer (Toronto Research Chemicals) was provided by the study authors via subsequent personal communication with the EPA. The procedures used were essentially the same as those used by the authors when testing the Artic char, *Salvelinus alpinus* (summarized below). Stock solutions

were prepared using dimethyl sulfoxide (DMSO) to achieve a final solvent concentration of 0.01% (v/v) during exposures. White sturgeon were from in-house cultures raised from embryos in the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan that originated from wild fish stock spawned at the Nechako White Sturgeon Conservation Centre, Vanderhoof, BC, Canada. Fish were cultured under flow-through conditions in facility water until they reached the juvenile stage (~4.5 years, 42.4 cm, 462.3 g). Prior to testing, fish were acclimated for 48-96 hours, during which they were fed commercial fish feed at a daily rate of 1% of body weight. Fish were exposed to only one nominal concentration (20 µg/L) that could be achieved using the limited amount of chemical available and that was nearing water solubility, while still being environmentally relevant. Test organisms were exposed in 700 L glass-fiber Min-o-Cool tanks containing 500 L of test solution at 12 ± 1 °C for 96 h under static-renewal conditions. Water was exchanged daily at 40-60% with a total of 12 fish exposed (three replicate tanks each for the control and 6PPD-q treatment with two fish per tank). Average observed water quality parameters were 12.8 ± 0.8 °C; pH of 8.35 ± 0.45; dissolved oxygen of 92.8 ± 13.2%; ammonia of 0.14 ± 0.15 mg/L and total hardness of 132 ± 6.80 mg/L. Water samples were collected for analytical confirmation of concentrations of 6PPD-q ~1 hour after the initial dosing of tanks. A water sample was also taken every 24 hours prior to water changes or after most fish in a tank became moribund. The average measured 6PPD-q concentration of the 20 µg/L nominal treatment water was 12.7 µg/L. Fish were observed during most of the exposure duration and were immediately removed once they became moribund. No mortalities were observed at the measured exposure concentration of 12.7 µg/L after 96 hours. The resultant 96-hour LC_{50} was >12.7 µg/L. This test was deemed qualitative since there was only one

exposure concentration and there were too few test organisms per treatment compared to the EPA's 850 Ecological Effects Test Guidelines.

E.1.1.4 Genus Oncorhynchus

Tian et al. (2021) evaluated the 24-hour toxicity of N-(1,3-dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, ≈98% purity) to coho salmon (*Oncorhynchus kisutch*). Initially, purified 6PPD-q was obtained via ozonation of industrial grade (96% purity) 6PPD (N-(1,3-dimethylbutyl)-N′ -phenyl-p-phenylenediamine). Coho salmon from Soos Creek or Diru Creek stock were reared at the Puyallup Research and Extension Center of Washington State University on a 12:12 h light:dark cycle in a custom recirculating water system and fed commercial food. Test water was dechlorinated municipal water treated by reverse osmosis to Type 3 (< 0.25 uS/cm) in a Rios 200 purification system (Millipore Sigma) and then reconstituted with buffered Instant Ocean salts to approximately pH 7.5 and 1,300 uS/cm conductivity at 10 - 13 ºC. Individual coho salmon used in experiments were between age > 0 and 2 year (1.3 - 28.0 g); experimental results reflect replication across year classes and sources of fish, for each set of fish exposures. Aquaria were held in a recirculating water bath maintained at 10 - 12 ºC, 1,250 - 1,300 µS/cm specific conductivity, pH of 7.6 - 7.8, photoperiod of 12:12 light:dark, and aerated to maintain dissolved oxygen levels > 98% saturation. Individual aquaria contained 30 L of test solution. This biomass loading rate (between 0.38 g/L and 7.5 g/L) may have exceeded the EPA's 850 Ecological Effects Test Guidelines in some tanks (0.8 g/L; U.S.EPA 2016b). Twenty-four hour toxicity tests were performed using eight juvenile fish per concentration across 10 measured concentrations (0 [control], ~0.1 [control], ~0.2, ~0.3, ~0.5, ~0.8, ~1, > 1- < 2, ~2 ug/L, ~3, ~4 and ~5 µg/L). A solvent control (material and amount by volume not provided) and a positive control (250 mg/L TWP leachate) were additionally

included. Each concentration was replicated twice. No mortality was observed in the control, and 100% mortality was observed in the highest exposure concentration, resulting in a 24-hour LC_{50} of 0.79 µg/L for 6PPD-q. 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 5 hours and initial symptoms (locomotor deficiencies) evident within 90 minutes. It should be noted that the supplemental materials state a LC_{50} of 1.46 µg/L. The EPA reached out to the study authors to inquire about the differences between these values. The 24-hour LC_{50} of 0.79 µg/L 6PPD-q represents the measured data and the LC_{50} of 1.46 µg 6PPD-q/L in the supplemental materials represents the data in nominal concentrations, to be consistent with the other data (6PPD) presented graphically in the paper. Additionally, the study authors noted, in a subsequent erratum, that a similar follow-up experiment was conducted using commercially available 6PPD-q. Results of the follow up study are described in Tian et al. (2022). The EPA considered the 24-hour LC_{50} of 0.79 µg/L 6PPD-q from measured concentrations acceptable for qualitative use because the exposure concentration was only 24 hours, as opposed to the recommended test duration of 96 hours in the EPA's 850 Ecological Effects Test Guidelines (U.S.EPA 2016b), and, there appear to have been issues with biomass loading. The LC_{50} of 1.46 µg 6PPD-q/L based on nominal concentrations was not considered further. The 24-hour LC_{50} of 0.79 µg 6PPD-q/L originally stated in the paper was amended in an errata to the original article, which stated that the latest LC_{50} was 0.095 µg 6PPD-q/L with the use of a commercial standard of 6PPD-q. This later value is presented in greater detail in Tian et al. (2022) and was used quantitatively to derive the screening value (see Section 3.1.1.1). The two concerns noted above

together with the change in the LC_{50} value presented in the paper resulted in the qualitative use of the original study and corresponding LC_{50} value of 0.79 µg 6PPD-q/L.

The acute effects of N-(1,3-Dimethylbutyl)-N'-phenyl-p-phenylenediamine-quinone (6PPD-q, CAS# 2754428-18-5, purity > 95%, purchased from Cambridge Isotope Laboratories) to the Japanese salmon, *Oncorhynchus masou ssp. masou,* was investigated by **Hiki and Yamamoto (2022)**, with the methodology (static-renewal, measured exposure) very similar to that used by the authors when testing Southern Dolly Varden (*Salvelinus curilus*), summarized in Section 4.2.1.1.2. Fish (all < 1 year old) were from the Taisetsu fishing industry industrial association (Kamikawa, Hokkaido, Japan). Test organisms were maintained using dechlorinated tap water (pH 7.2, hardness of 68 mg/L and conductivity of 22.3 mS/m) in a commercial recirculating water system at the National Institute for Environmental Studies for more than 2 weeks at 14 ºC under a 12:12 hour light-dark cycle. Fish were fed with a commercial fish food three times per week. Acute static-renewal measured lethality tests were performed according to the OECD Test Guideline 203 with slight modifications that are noted below and were determined to be inconsequential. Four juvenile fish (with each individual fish weighing an average of 6.7 g and with an average length of 9.3 cm; personal communication with Kyoshiro Hiki, March 2023) were exposed for 96 hours in a glass tank containing 5 L of dechlorinated tap water spiked with 10 µg/L 6PPD-q. The carrier solvent control was dosed with acetonitrile at 0.012% (v/v). Test chambers were 19 cm x 19 cm x 20 cm glass tanks. This experimental design resulted in a biomass loading rate of 5.36 g/L. This biomass loading rate was six times higher than the recommendation of 0.8 g/L in the EPA's 850 Ecological Effects Test Guidelines (U.S.EPA 2016b). The tanks were kept in a temperature-controlled room at 14 ºC under a 12:12 hour light-dark photoperiod and aerated through a Pasteur pipet connected with an air pump. The

temperature, oxygen, and pH in test solutions were checked daily and were 14.4 ± 0.3 ºC, > 60% saturation, and 7.5 ± 0.3, respectively. Additional food was not provided. After 48 hours, surviving fish were transferred to a glass tank containing 5 L of a newly prepared test solution to keep exposure concentrations stable. The measured 6PPD-q concentrations were 54 − 109% of the nominal ones just after water renewal, while 47 − 97% of the detected 6PPD-q was lost after 48 hours of water renewal. No lethality or abnormal behavior of fish was observed during the exposure duration. Thus, the 96-hour LC_{50} was >3.5 µg/L 6PPD-q (based on the time weighted average measured concentration). This study was deemed qualitatively acceptable because there was only one concentration tested, which resulted in a greater than low value, and only four fish per treatment without replication.

E.1.1.5 Genus Salmo

Foldvik et al. (2022) investigated the acute toxicity of N-(1,3-Dimethylbutyl)-N'-phenyl-p-phenylenediamine-quinone (6PPD-q, CAS# 2754428-18-5, purity not reported, purchased from Cambridge Isotope Laboratories) to the Atlantic salmon, *Salmo salar* in a static, measured exposure. The 6PPD-q stock was dissolved in acetone (100 mg/L), evaporated using a flow of nitrogen, and then the residue was dissolved in fish medium to create an initial stock solution. The stock solution was then serially diluted to produce target exposure (nominal) concentrations of 12.2, 6.08, 3.04, 1.52, 0.760, 0.380, 0.190 and 0.095 µg 6PPD-q/L. The fish used in the experiment were alevins (average weight of 0.111 g) obtained from the research station stock (Norwegian Institute for Nature Research, Aquatic Research Station, Rogaland, Norway). Dilution water consisted of nearby lake water that was ozone treated, 0.1 mm filtered, UV treated, and oxygenated to 100% saturation. Average water quality characteristics during testing were 4.7 ºC, pH 6.4, turbidity 0.36 FNU, conductivity 7.3 mS/m, dissolved oxygen 10.5 mg/L,

and total organic carbon 3.0 mg/L. Fish were gently transferred from hatchery tanks to ~1.5 L glass containers (10-13 fish per tank) containing 1 L test solution and statically exposed for 48 hours to two water only controls and eight measured exposure concentrations of 6PPD-q (0, 0, 0.095, 0.19, 0.38, 0.76, 1.52, 3.04, 6.08 and 5.75 µg/L), with one test chamber per treatment. Concentrations of 6PPD-quinone decreased substantially during the 48-hour exposure and were on average 28% of the initial concentrations after 48 hours. The 6PPD-q concentration in the highest treatment with an initial concentration of 12.16 µg/L decreased to 5.75 µg/L after 48 hours. Fish were observed every 3-7 h throughout the experiment, and fish that were not actively moving were gently touched on the caudal peduncle to produce a reaction. No mortality or abnormal behavior was observed during the experiment. The authors reported a LC_{50} of >5.75 µg 6PPD-q/L. However, this study was classified as qualitative because exposure concentrations were not replicated.

Foldvik et al. (2022) also evaluated the static, measured acute sensitivity of *Salmo trutta* (brown trout) to of N-(1,3-Dimethylbutyl)-N'-phenyl-p-phenylenediamine-quinone (6PPD-q, CAS# 2754428-18-5, purity not reported, purchased from Cambridge Isotope Laboratories). Test procedures were essentially the same as used by the authors for *S. salar*. The 6PPD-q stock was dissolved in acetone (100 mg/L), evaporated using a flow of nitrogen, and then the residue was dissolved in fish medium to create an initial stock solution. The stock solution was then serially diluted to produce target exposure (nominal) concentrations of 12.2, 6.08, 3.04, 1.52, 0.760, 0.380, 0.190 and 0.095 µg/L 6PPD-q. The fish used in the experiment were alevins (average weight of 0.076 g) obtained from the research station stock (Norwegian Institute for Nature Research, Aquatic Research Station, Rogaland, Norway). Dilution water consisted of nearby lake water that was ozone treated, 0.1 mm filtered, UV treated, and oxygenated to 100% saturation.

Water quality characteristics during testing were 4.7 ºC, pH 6.9, turbidity 0.36 FNU, conductivity 7.3 mS/m and total organic carbon 3.0 mg/L. Fish were gently transferred from hatchery tanks to ~1.5 L glass containers (10-13 fish per tank) containing 1 L test solution and statically exposed for 48 hours to two water only controls and eight measured exposure concentrations of 6PPD-q (0, 0, 0.095, 0.19, 0.38, 0.76, 1.52, 3.04, 6.08 and 8.98 µg/L), with one test chamber per treatment. The 6PPD-q concentration in the highest treatment with an initial concentration of 12.16 µg/L decreased to 8.98 µg/L after 48 hours. Fish were observed every 3-7 h throughout the experiment, and fish that were not actively moving were gently touched on the caudal peduncle to produce a reaction. No mortality or abnormal behavior was observed during the experiment. The authors reported a LC_{50} of >8.98 µg 6PPD-q/L. However, this study was classified as qualitative because exposure concentrations were not replicated, there were only 10 to 13 fish per treatment concentration, and the test duration was too short compared to the EPA's 850 Ecological Effects Test Guidelines.

E.1.1.6 Genus Salvelinus

Hiki and Yamamoto (2022) also evaluated the acute toxicity of 6PPD-q to the Southern Dolly Varden, *Salvelinus curilus*, using a test methodology (static-renewal, measured exposure) that was similar to the quantitative study on whitespotted char (*Salvelinus leucomaenis ssp. pluvius*) summarized in Section 3.1.1.2. The 6PPD-q (CAS# 2754428-18-5, purity > 95%) was purchased from Cambridge Isotope Laboratories and dissolved in acetonitrile. Fish acclimation and testing was conducted at 14 ºC under a 12:12 hour light-dark photoperiod with aeration provided by a Pasteur pipet/air pump. Prior to testing, fish were fed with a commercial fish food three times per week and maintained in dechlorinated tap water at pH 7.2, hardness of 68 mg/L, and conductivity of 22.3 mS/m. Acute static-renewal measured lethality tests were performed

according to the OECD Test Guideline 203 with slight modifications that are noted below and were determined to be inconsequential. Four juvenile, fish all < 1 year old (each individual fish weighing an average of 3.6 g and with an average length of 8.2 cm; personal communication with Kyoshiro Hiki, March 2023) were exposed for 96 hours in a 19 cm x 19 cm x 20 cm glass tank containing approximately 5 L of dechlorinated tap water spiked with 10 µg 6PPD-q/L. This experimental design resulted in a biomass loading rate of 2.88 g/L, which was over three times higher than the recommendation of 0.8 g/L in the EPA's 850 Ecological Effects Test Guidelines (U.S.EPA 2016b). The carrier solvent control was dosed with acetonitrile at 0.012% (v/v). The temperature, oxygen, and pH in test solutions were checked daily and were 14.4 ± 0.3 ºC, > 60% saturation, and 7.5 ± 0.3, respectively. Food was not provided during the test. After 48 hours surviving fish were transferred to a glass tank containing 5 L of a newly prepared test solution to keep exposure concentrations stable. The measured 6PPD-q concentrations were 54 – 109% of nominal just after water renewal, while 47 – 97% of the detected 6PPD-q was lost after 48 hours of water renewal. No lethality or abnormal behavior of fish was observed during the exposure duration. Thus, the 96-hour LC_{50} was > 3.8 µg/L 6PPD-q (based on the time weighted average measured concentration). This study was deemed qualitative because there was only one concentration tested. Additionally, there were only four fish per treatment without replication whereas the EPA'S 850 Ecological Effects Test Guidelines states that a minimum of seven fish are required and that 10 fish and two replicates per treatment level is preferred to obtain a more statistically accurate representation of the concentration-response curve (U.S.EPA 2016b). This author-reported LC_{50} of > 3.8 µg 6PPD-q/L was used qualitatively as supporting information and indicates that Southern Dolly Varden (*Salvelinus curilus*) would be protected by the acute freshwater screening value for 6PPD-q.

The sensitivity of the Artic char, *Salvelinus alpinus*, to 6PPD-q was evaluated by **Brinkmann et al. (2022)** via a 96-hour static-renewal, measured toxicity test. N-(1,3-Dimethylbutyl)-N′-phenyl-p-phenylenediamine-quinone (6PPD-q, CAS# 2754428-18-5). Chemical purity was not reported in the paper, but a purity of 97% and the name of the manufacturer (Toronto Research Chemicals) was provided by the study authors via subsequent personal communication with the EPA. Stock solutions were prepared using dimethyl sulfoxide (DMSO) to achieve a final solvent concentration of 0.01% (v/v) during exposures. Arctic char were from in-house cultures raised from embryos at the Aquatic Toxicology Research Facility at the University of Saskatchewan, that originated from fish stock obtained from Miracle Springs Inc. (North Vancouver, BC). Fish were cultured under flow-through conditions in research facility water until they reached the juvenile stage (~3 years, 13.8 ± 1.7 cm, 28.3 ± 9.8 g). Prior to testing, fish were acclimated for 48 - 96 hours, during which they were fed commercial fish feed at a daily rate of 1% of body weight. Fish were exposed to only one nominal concentration (20 μg/L) that could be achieved using the limited amount of chemical available and that was nearing water solubility, while still being environmentally relevant. Test organisms were exposed in 700 L glass-fiber Min-o-Cool™ tanks containing 300 L of test solution at 12 ± 1 °C for 96 hours under static-renewal conditions. Test solutions were renewed (75% renewal) daily with a total of 20 fish exposed (two replicate tanks each for the solvent control (0.01% (v/v) DMSO) and 6PPD-q treatment with five fish per tank). These conditions resulted in a biomass loading rate of 0.47 g/L, which is well within the EPA's 850 Ecological Effects Test Guidelines of 0.8 g/L (U.S.EPA 2016b). Average observed water quality parameters were 12.8 ± 0.8 °C; pH of 8.35 ± 0.45; dissolved oxygen of 92.8 ± 13.2%; ammonia of 0.14 ± 0.15 mg/L and total hardness of 132 ± 6.80 mg/L. Water samples were collected for analytical confirmation of

concentrations of 6PPD-q ~1 hour after the initial dosing of tanks. A water sample was also taken every 24 hours prior to water changes or after most fish in a tank became moribund. The average measured 6PPD-q concentration of the 20 µg/L nominal treatment water was 14.2 µg/L. Fish were observed during most of the exposure duration and were immediately removed once they became moribund. No mortalities were observed at the measured exposure concentration of 14.2 µg 6PPD-q/L after 96 hours. The resultant 96-hour LC_{50} was >14.2 µg 6PPD-q/L. This test was deemed qualitative since there was only one exposure concentration.

E.1.1.7 Danio rerio

Hiki et al. (2021) performed a 96-hour static-renewal, measured test of N-(1,3-dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, 83.6 ± 8.9% purity, synthesized in the laboratory via stepwise Michael addition of aniline and 1,3-dimethylbutylamine to 1,4-benzoquinone) 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 (details about the use of solvent control were not provided) and a single treatment (54 µg/L measured 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 31.5 mS/m, respectively. The photoperiod was 16 hours light and 8 hours dark. The study authors did not report a comparison of measured concentrations to nominal in order to track potential fluctuations of 6PPD-q over the exposure

duration. No mortality was observed in the control or test concentration resulting in a 96-hour LOEC of >54 µg 6PPD-q/L. It was considered acceptable for qualitative use because there was only one test concentration, which resulted in a low greater than LOEC (U.S.EPA 2013).

Ji et al. (2022) performed a 12-hour static measured test of N-(1,3-dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, CAS# 2754428-18-5, 98.0% purity, obtained from Jiaxuan Biotechnology Co., Ltd. (Hengyang, 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 5,000 mg/L stock solution of 6PPD-q 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-q), 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. This experimental design resulted in a biomass loading rate of 0.3 g/L, which is within the recommended loading rate of 0.8 g/L (U.S.EPA 2016a). Fish were not fed for 24 hours prior to the experiment or during the experiment. The water temperature during the experiment was 26°C. The study authors report that 6PPD-q concentration was stable over the exposure duration of 12 hours, with chemical measurements taken every two hours and displaying stability of 6PPD-q. No statistically significant differences ($P > 0.05$) in swimming velocity or distance were observed during the experiment, resulting in a 12-hour NOEC of >1,000 µg 6PPD-q/L. The test was considered qualitative because it was of insufficient duration (12 hours as opposed to the 96

hours preferred in the EPA's 850 Ecological Effects Test Guidelines (U.S.EPA 2016b) or 24 hours that is commonly used in other 6PPD-q toxicity studies summarized in this document) for an acute toxicity test and was based on a non-apical behavioral endpoint.

Zhang et al. (2023) conducted a 112-hour static-renewal, unmeasured acute test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, >98% purity, obtained from Toronto Research Chemicals (Toronto, Canada)) 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 h (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-q 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 solvent control animals also received 0.1% DMSO (v/v in embryo medium). Zebrafish embryos at 8 hpf were continuously exposed to 6PPD-q during the embryo development stage until 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 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 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-q/L, which was acceptable for qualitative use due to atypical test duration.

E.1.1.8 Oryzias latipes

Hiki et al. (2021) performed a 96-hour static-renewal measured test of N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine quinone (6PPD-q, 83.6±8.9% purity, synthesized in the laboratory via stepwise Michael addition of aniline and 1,3- dimethylbutylamine to 1,4-benzoquinone) 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 (34 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 (details about the use of solvent control not provided) and treatment. Dissolved oxygen, pH, temperature, and conductivity averaged 7.95 mg/L, 7.9, 24.4 °C, and 34.3 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 >34 µg/L. The LC_{50} was considered acceptable for qualitative use because test treatments chambers were not replicated and because there was only one test concentration.

E.2 Summary of Estuarine/Marine Qualitative Acute Toxicity Data

Species (lifestage)	Method[a]	Test Duration	Chemical / Purity	pH	Temp. (°C)	Salinity (ppt)	Effect	Reported Effect Concentration (µg/L)	Deficiencies	Reference
Rotifer, *Brachionus koreanus*	R, U	24 hr	6PPD-q Not provided	-	25	30	LC50	>1,000	Lack of exposure details	Maji et al. (2023)

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

E.2.1 Summary of Acute 6PPD-q Toxicity Studies Considered Qualitatively in the Estuarine/Marine Screening Value Derivation

E.2.1.1 Brachionus koreanus

Maji et al. (2023) conducted a 7-day unmeasured, static-renewal chronic test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-Q, purity not provided, synthesized by ASCA GmbH - Berlin, Germany)) with the marine rotifer, *Brachionus koreanus*. While the test details for the 7-day test are provided in the publication, little to no details are provided for the 24-hour acute exposure. Therefore, the accompanying 24-hour LC_{50} of >1,000 µg 6PPD-Q/L was classified as qualitative due to lack of exposure details.

Appendix F Chronic Qualitative Toxicity Data

F.1 Summary of Freshwater Qualitative Toxicity Data

Species (lifestage)	Method[a]	Test Duration	Chemical / Purity	pH	Temp. (°C)	Hardness (mg/L as CaCO3)	Effect	Reported Effect Concentration (µg/L)	Deficiencies	Reference
Washboard mussel (~7 yr), *Megalonaias nervosa*	R, M	8 d	6PPD-q >99.8%	7.2-7.6	25	105.5	NOEC (mortality)	11.4	Atypical test duration; too few organisms per exposure treatment (n=4)	Prosser et al. (2023)
Washboard mussel (~7 yr), *Megalonaias nervosa*	R, M	8 d	6PPD-q >99.8%	7.2-7.6	25	105.5	NOEC (mortality)	17.9	Atypical test duration; too few organisms per exposure treatment (n=4)	Prosser et al. (2023)
Coho salmon (embryo), *Oncorhynchus kisutch*	S, U	4 x 24 hr pulses	6PPD-q 97.26%	-	8.5	-	LOEC (mortality at 6 dph)	7.22	24 hour pulsed exposures over a 14 day exposure period	Greer et al. (2023b)

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

F.1.1 Summary of Chronic 6PPD-q Toxicity Studies Considered Qualitatively in the Freshwater Screening Value Derivation

F.1.1.1 Megalonaias nervosa

Prosser et al. (2023) conducted an 8-day static-renewal, measured test of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, >99.8% purity, purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada)) with the washboard mussel, *Megalonaias nervosa*. Test organisms were obtained from Dr. Christopher Barnhart's laboratory at Missouri State University. The mussels were cultured in the laboratory from glochidia (larvae) that had been collected from the wild. The mussels were approximately seven years of age. Upon arriving at the University of Guelph's Hagen Aquatic Science Laboratory, the mussels were given a week to acclimate to conditions in the laboratory before any experimentation was initiated. No mortality was observed due to shipping from Missouri or during the acclimatization period. For the experiments, individual mussels were placed into a 3 L glass beaker (151 mm diameter x 198 mm height) with roughly 5 cm of sand placed at the bottom of the beaker inside of a growth chamber set to 25 ℃ with a 16:8 hour light:dark cycle. Each beaker was then filled with 2 L of dechlorinated City of Guelph drinking water. Each jar was individually spiked with a volume of concentrated stock solution that was prepared by dissolving 6PPD-q in HPLC-grade methanol. The experiment with *M. nervosa* was performed twice (experiment A and B). The exposure concentrations used in experiment A were 2.5, 5, 10, and 20 µg/L, along with a negative control and a solvent control (0.02% solvent by volume). Each treatment contained four replicates as well as a laboratory blank test vessel to provide a sample of exposure water for the confirmation of the concentration of 6PPD-q. The exposure concentrations in experiment B were 12.5, 25, and 50 µg/L along with a negative control and solvent control (0.02% solvent by volume). Each test vessel was aerated using an airline with a Pasteur pipette at the end. A 50%

water change was conducted each day of the test. The water removed on day 2 and 3 was collected and frozen at -20 ℃ until analyzed to measure the concentration of 6PPD-q (quantified using solid phase extraction followed by ultraperformance liquid chromatography (UPLC) and tandem mass spectrometry (MS-MS). The mussels were fed 4 mL of a mixture of 0.7% Nanno® and 2.7% Shellfish Diet® (Reed Mariculture) daily. Mortality of mussels was monitored daily throughout the test. Surviving mussels were returned to isolated culture conditions to monitor their health over a 90-day period. Exposure solutions were sampled when they were made on day 0 in tests A and B, while samples were taken on days 2 and 3 during test B before the 50% water changes to document the decline in the concentration of 6PPD-q over the course of the exposure. Water chemistry measurements of pH, dissolved oxygen, ammonia and temperature were taken at the initiation and completion of the experiment and ranged from 7.2-7.6 SU, 7.98-8.15 mg/L, 0-0.25 mg/L and 25 ℃, respectively. The measured concentration of 6PPD-q was consistently lower than the nominal concentration. The percent difference between measured and nominal concentration at day 0 ranged from 6.0% to 43.0% for Test A, and from 49.2% to 69.5% for Test B. Control organism survival was 100% for both tests in the water only and solvent controls. The 8-day NOEC for mortality was 11.4 µg 6PPD-q/L for Test A, and 17.9 µg 6PPD-q/L for Test B. Data from both tests were acceptable for qualitative use due to atypical test duration and too few organisms per exposure treatment (n=4).

F.1.1.2 Oncorhynchus kisutch

Greer et al. (2023b) conducted a 14-day exposure consisting of four 24-hour pulsed measured, static acute tests per week of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, 97.26% purity, obtained from HPC Standards) with the coho salmon, *Oncorhynchus kisutch*. Fertilized embryos at the eyed egg stage used for testing were obtained

from the Issaquah Salmon Hatchery (Issaquah, WA). Eggs were transported to the U.S. Geological Survey Western Fisheries Research Center in Seattle, WA, and reared in egg stacks with spinning disc filtered (2 µM final) and ultraviolet-treated freshwater from Lake Washington (100 µS) maintained at 8.0 - 8.5 °C. Embryos were acclimated for a minimum of 96 hours before initial exposure. 6PPD-q was dissolved in dimethyl sulfoxide (DMSO) to make a concentrated stock solution. A working 1X stock solution of 10 µg/L 6PPD-q in process water was prepared immediately prior to the start of exposure and serial diluted to obtain 1X working solutions of 1.0 and 0.1 µg/L. Four static 24-hour pulse exposures to nominal 6PPD-q concentrations of 0.1, 1 or 10 µg/L or a DMSO solvent carrier control were carried out in glass Petri dishes (150 mm x 20 mm) containing 170 mL of water at a density of 20 embryos per dish. Each exposure period covered eight accumulated temperature units (ATUs) of embryonic development. Dishes were placed at the bottom of circular 2-foot tanks and surrounded by low-flow temperature-controlled water to maintain 8.5 °C in exposure dishes. Embryos were exposed to four 24 hour pulses of 6PPD-q or solvent carrier control (volume not provided) twice per week until hatch (six replicate dishes, n = 120 embryos/concentration) to mimic intermittent winter rain events. After each 24-hour exposure, eggs were carefully transferred back to egg rearing trays (1 tray per treatment group) and maintained in clean flowing water. Embryos that hatched prior to the final exposure were excluded from subsequent exposures but continued to be monitored for mortality. Surviving, unhatched embryos were randomly distributed to the dishes for the next exposure. Mortality and hatching were monitored daily throughout the experiment, with hatching defined as the complete liberation of both the head and tail from the chorion. Embryos exhibiting abnormal phenotypes were monitored for a minimum of 24 hours to ensure the embryo was not in the process of a successful hatch. The larvae assessed in the experiment were collected

directly from the glass exposure dishes following the fourth exposure in which most embryos hatched to ensure that all larvae measured were less than 24 hours post hatch. No mortality was observed in any solvent control treatments. Concentrations of 6PPD-q were determined by direct injection ultraperformance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS). Duplicate exposure samples were collected in 4 mL amber glass vials and stored at -20 °C until analysis. 6PPD-q initial and final concentrations were assessed from the second and fourth exposures and the initial concentration from the third exposure. Photoperiod and light intensity were not reported. No effects were observed after the first 24-hour pulse. Effects at the highest test concentration started to occur after the second 24-hour exposure. The author-reported 14-day LOEC for mortality at 6 dph of eyed embryos was 7.22 µg 6PPD-q/L, which was acceptable for qualitative use, because of the atypical test design.

F.2 Summary of Estuarine/Marine Chronic Qualitative Toxicity Data

No data

Appendix G Quantitative Freshwater Plant Toxicity Data

G.1 Summary Table of Acceptable Quantitative Freshwater Plant 6PPD-q Toxicity Studies

Species (lifestage)	Method[a]	Test Duration	Chemical / Purity	pH	Temp. (°C)	Effect	Author Reported Effect Conc. (µg/L)	Reference
Green alga, *Chlamydomonas reinhardtii*	S, U	72 hr	6PPD-q Not reported	-	20	LOEC (population growth rate)	250	Wu et al. 2023

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

G.1.1 Summary of Plant 6PPD-q Toxicity Studies Considered Quantitatively in the Freshwater Screening Value Derivation

G.1.1.1 Chlamydomonas reinhardtii

Wu et al. (2023) evaluated the 72-hour toxicity of N-(1,3-Dimethylbutyl)-N′ -phenyl-p-phenylenediamine quinone (6PPD-q, purity not provided, synthesized in the laboratory) with the green algae, *Chlamydomonas reinhardtii* in a static, unmeasured exposure. Wild-type *C. reinhardtii* (strain UTEX 90) was obtained from the University of Texas Algal Collection. The strain was maintained in a TAP medium in an environmental chamber with a 12:12 hour light:dark cycle. The temperature of the chamber was kept at 20 ±1°C and the light intensity was set as 120W/m^2. The experiment was carried out under the same condition as the maintenance of the strain in the environmental chamber. 6PPD-q exposures were conducted in triplicate in 15 flasks containing 5 mL of TAP medium with final nominal concentrations of 0, 250 and 1,000 µg/L for 72 hours. The use of solvent during testing was not provided. At test termination, microalgae were collected by centrifugation at 13,000 rpm for 5 min for further analysis. The author-reported 72-hour population growth rate LOEC was 250 µg 6PPD-q/L, which was acceptable for quantitative use.

Appendix H Unused Toxicity Data

Author	Citation	Reason Unused
Mahoney, H., F.C. Da Silva Junior, C. Roberts, M. Schultz, X. Ji, A.J. Alcaraz, D. Montgomery, S. Selinger, J.K. Challis	2022. Exposure to the Tire Rubber-Derived Contaminant 6PPD-quinone Causes Mitochondrial Dysfunction In Vitro. Environ. Sci. Technol. Lett.9(9): 765-771.	*In vitro* exposures on excised cells (not whole body)