1 # Main Manuscript for

2 ## Structurally Selective Ozonolysis of *p*-Phenylenediamines and Toxicity in

3 ## Coho Salmon and Rainbow Trout

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Author Contributions

# L.X. and J.Y. contributed equally to this work.

L.X., J.Y., J.A., and H.P. designed research; L.X., J.Y., P.N., J.S., B.H., M.O., X.Q., and D.Y.

performed research; L.K., D.K., S.K., C.H., J.H., and J.G. contributed to fish and cell line

experiments; L.X. and J.Y. analyzed data; L.X., J.Y., J.A., and H.P. wrote the paper.

This manuscript includes:

    Main Text

    Figures 1 to 6

    Scheme 1

Abstract

The tire rubber-derived ozonation product of *N*-(1,3-dimethylbutyl)-*N′*-phenyl-*p*-phenylenediamine (6PPD), *N*-(1,3-dimethylbutyl)-*N′*-phenyl-*p*-phenylenediamine-quinone (6PPD-Q), was recently discovered to cause acute mortality in coho salmon (*Oncorhynchus kisutch*). *para*-Phenylenediamines (PPDs) with variable side chains distinct from 6PPD have been identified as potential replacement commercial antioxidants, but their structure-related ozone reactivities and toxicities remain unexplored. We herein tested the multiphase gas-surface ozone reactivity of four select PPDs and evaluated the toxicity of their reaction mixtures in coho salmon and rainbow trout (*Oncorhynchus mykiss*). 6PPD and *N*-Isopropyl-*N′*-phenyl-*p*-phenylenediamine (IPPD) were found to rapidly react with ozone to form 22 and 16 transformation products, respectively, including PPD-Qs. No significant multiphase ozone reactivity was observed for *N,N′*-Diphenyl-*p*-phenylenediamine (DPPD) or *N*-Cyclohexyl-*N′*-phenyl-*p*-phenylenediamine (CPPD) despite their structural similarity to 6PPD. The viability of coho salmon CSE-119 cells was strongly affected by the ozonolysis products of 6PPD, but not by those of the other three PPDs. The cytotoxicity of the 6PPD reaction mixture increased with ozonolysis time, with the strongest toxicity being observed after 7 days of oxidation by 100 ppb of ozone. As with coho salmon cells, acute mortality was only observed in juvenile rainbow trout that were exposed to the oxidized 6PPD reaction mixture, suggesting a common mechanism of toxic action in the two salmonid fish species. Compound- and regio-selective formation of hydroxylated metabolites of 6PPD-Q were detected in rainbow trout exposed to the 6PPD reaction mixture, which may be related to its selective toxicity. This study reports the structurally selective ozone reactivity of PPDs, and the unique toxicity of 6PPD ozonolysis mixtures, which demonstrates that other PPDs are potential alternative antioxidants.

Significance Statement

The development of alternative antioxidants to replace 6PPD is needed. This study reports the unexpectedly selective ozone reactivity of four PPDs, and the unique toxicity of oxidized 6PPD

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65 reaction mixtures compared to those of other PPDs, despite their structural similarities. This

66 demonstrates it is possible for alternative PPDs to replace 6PPD in the future.

Introduction

Coho salmon (*Oncorhynchus kisutch*) are one of seven *Oncorhynchus* fish species native to North America. Importantly, specific coho salmon populations have been declining since the 1970s including four distinct populations that are listed as endangered or threatened (1-3). In urbanized watersheds, 40-90% of returning coho salmon die before spawning, a phenomenon known as 'urban runoff mortality syndrome' (4). A recent milestone study identified the ozonation product of *N*-(1,3-dimethylbutyl)-*N'*-phenyl-*p*-phenylenediamine (6PPD), *N*-(1,3-dimethylbutyl)-*N'*-phenyl-*p*-phenylenediamine-quinone (6PPD-Q), as the toxicant responsible for the acute mortality of coho salmon (5). 6PPD-Q exerts extreme toxicity in coho salmon, with a median lethal concentration (LC_{50}) of 0.095 µg/L (6). 6PPD-Q also exerts toxicity to several other salmonid fish species including rainbow trout (*Oncorhynchus mykiss*) with large interspecies variation in lethality (7-9), but the toxicity mechanism remains unclear. The reaction of 6PPD with atmospheric ozone is generally considered to be the major pathway for the formation of 6PPD-Q. Previous studies demonstrated the generation of 6PPD-Q from homogeneous ozonation of 6PPD in phosphate buffer solution (10), and the heterogeneous gas-phase ozonation of pure solid 6PPD (11) and 6PPD present in tire tread wear particles (12). 6PPD has been widely used as an antioxidant to protect rubber tires from oxidative aging where transport of 6PPD-Q typically occurs from ozonation of tire wear particles and dust that ultimately reach aquatic ecosystems through stormwater runoff (13). Globally, approximately 3.1 billion car tires are manufactured annually, with the United States alone consuming 6PPD in the range of 50 to 100 million pounds each year (14). Not surprisingly, 6PPD-Q and 6PPD have been widely detected in surface waters across Canada and other countries (15-17), underscoring the pervasive exposure to salmonid fish worldwide during freshwater residence. There is a pressing need to develop safe alternative antioxidants to replace 6PPD.

The development of alternative antioxidants to 6PPD is challenging for the following reasons: 1) It is not straightforward to identify compounds with greater ozone reactivity than alkene compounds, which is the major component of rubber products (14); 2) The alternative antioxidants must demonstrate excellent diffusivity in rubber to ensure persistent surface protection over time; 3) The

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94 toxicities of alternative antioxidants are difficult to predict. Therefore, instead of developing a new

95 class of antioxidants, the ideal case is to further refine the existing PPD compound class to

96 determine less toxic alternatives. Indeed, our recent study reported the selective toxicity of 6PPD-

97 Q amongst seven structural analogues of PPD-Qs (18). However, even though the preliminary

98 results implied the potential for other PPDs to be applied as substitutes of 6PPD, their safety was

99 evaluated for only PPD-quinones. As antioxidants to protect rubber from oxidation, PPDs can be

100 rapidly transformed by ozone to a variety of chemical species. Over 30 structurally diverse

101 transformation products have been reported to form via the reaction of 6PPD with ozone (10, 11).

102 Such chemical complexity in transformation products as well as the high toxicity observed for the

103 major product 6PPD-Q highlights the need to systematically assess the toxicity of all PPD

104 transformation products under environmentally relevant ozone conditions.

105 In this study, we aimed to systematically assess the structure-related ozone reactivity and toxicity

106 of four select PPDs including 6PPD, *N*-Isopropyl-*N'*-phenyl-*p*-phenylenediamine (IPPD), *N*-

107 Cyclohexyl-*N'*-phenyl-*p*-phenylenediamine (CPPD), and *N,N'*-Diphenyl-*p*-phenylenediamine

108 (DPPD). These compounds serve as representative PPDs with distinct alkyl, cycloalkyl, and aryl

109 side chains, informing the structure-related effects on ozone reactivity and toxicity of PPDs. In

110 addition, these PPDs are widely employed as commercial antioxidants, making them convenient

111 substitutes for 6PPD if they exhibit reduced toxicity. To achieve this, 1) we conducted multiphase

112 ozonolysis of PPDs using a flow reactor; 2) the structure-related toxicities of PPD ozonolysis

113 mixtures were evaluated in an embryonic coho salmon cell line; 3) the PPD ozonolysis mixtures

114 were dosed to juvenile rainbow trout for *in vivo* toxicity testing (Fig. 1).

115

116 **Results and Discussion**

117 **Multiphase ozonolysis of PPDs.** Multiphase gas-surface ozonolysis experiments were performed

118 in a flow reactor to simulate the transformation of PPDs in tire-wear particles when exposed to

119 atmospheric oxidants (10, 11). Preliminary results showed that with a level of 1 ppm of ozone,

120 approximately 60% of 100 μg and 50% of 500 μg of 6PPD were consumed after 24 hours (Fig.

121 S1A-B), which represented a notably fast consumption rate. Therefore, we opted to use a more

122 environmentally relevant level of ozone (100 ppb) for the reaction. Under the conditions of a starting

123 mass of 500 µg 6PPD and an ozone concentration of 100 ppb, >50% of 6PPD was oxidized within

124 4 days (Fig. S1C). Thus, the ozonolysis was performed at 100 ppb for a variety of periods (0, 3,

125 and 7 days) to reflect the aging of PPDs under environmentally relevant conditions. Assuming the

126 typical outdoor ozone concentration is ~40 ppb in an urban city (*e.g.,* Toronto, Canada) (19) and

127 the amount of ozone exposure received by film samples in the flow reactor was linearly proportional

128 to exposure time and ozone concentration, then exposure for 3 days and 7 days is equivalent to

129 outdoor exposure times of approximately 1 and 2.5 weeks, respectively. Environmental ozonation

130 of 6PPD for periods of 1-3 weeks are common during the antecedent dry summers experienced in

131 habitats utilized by 6PPD-Q impacted species, and thus this ozonation regime may represent the

132 mixture of transformation products present in urban runoff.

133 As shown in Fig. 2A, a 64.5 ± 2.8% consumption of 6PPD was observed after 7 days of ozone

134 reaction (6PPD$_{7d}$). Correspondingly, 6PPD-Q was detected as a major transformation product, and

135 its concentration increased along with ozonolysis time. 6PPD-Q was not detected in the negative

136 controls with zero air at the same setting (Fig. S2), clearly demonstrating its formation was due to

137 the reaction with ozone. The oxidation yield of 6PPD-Q at day 7 was 8.4 ± 0.7%, which was

138 comparable to that (10 ± 5%) observed by Zhao et al. (11).

139 To systematically characterize the reaction products of 6PPD, high-resolution mass spectrometry

140 (HRMS)-based nontargeted analysis was employed. Twenty-two LC-MS features were detected

141 as tentative transformation products. These features showed significantly higher ($p < 0.05$) peak

142 abundances at day 3 and 7 in comparison to day 0 (Fig. 2B), supporting their identities as

143 ozonolysis products. The identities of 4-Hydroxydiphenylamine (4-HDPA) and *N*-Phenyl-*p*-

144 phenylenediamine (4-ADPA) were confirmed by their authentic standards. 4-HDPA and 4-ADPA

145 might be formed through the oxidative *N*-dealkylation of 6PPD, followed by hydrolysis as reported

146 in a previous study (20). Similar to 6PPD-Q, increasing concentrations with ozonolysis time were

147 observed for both 4-HDPA and 4-ADPA (see Fig. S3A-B). The structures of 8 other transformation

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148 products were assigned based on their relative retention times to 6PPD and 6PPD-Q, their MS/MS

149 spectra (Fig. S4), and the reaction pathways reported in previous studies (10, 11). For the other 11

150 transformation products, their formulae were suggested according to *m/z* (Table S2). Additionally,

151 the concentrations of the 19 transformation products without commercial standards were semi-

152 quantified using the standard of 6PPD-Q, giving a total yield of 29.4 ± 5.9% after ozonolysis for 7

153 days (Fig. S3C). All transformation products account for approximately 65% of the 6PPD loss after

154 ozonolysis. Undetectable oligomers and volatile substances might contribute to the remaining

155 transformation products (18). These results demonstrate the formation of multiple structurally

156 diverse transformation products from the ozonolysis of 6PPD.

157 A similar consumption rate of IPPD (55.0 ± 14.8%) was observed after 7 days of ozonolysis (Fig.

158 2A), implying high ozonation potential for IPPD in consumer products. IPPD-Q, 4-ADPA and 4-

159 HDPA were also detected as the transformation products of IPPD, with 7-day reaction yields of

160 6.05 ± 1.59%, 5.54 ± 1.91%, and 0.40 ± 0.17%, respectively (Fig. 2A, S3A, S3B). Nontargeted

161 analysis revealed the formation of 16 tentative transformation products from the ozonolysis of IPPD

162 (Fig. S3C). In addition to IPPD-Q, 4-ADPA and 4-HDPA, five other transformation products shared

163 similar reaction pathways with those of 6PPD (Fig. S5), and 8 transformation products (Table S3)

164 from other pathways were also detected, the total yield of which was 14.6 ± 5.2% (Fig. S3E).

165 In contrast to 6PPD and IPPD with alkyl side chains, no significant oxidative consumption was

166 observed for CPPD or DPPD with cycloalkyl and aryl side chains (Fig. 2A). Consistent with this, no

167 significant transformation products were detected from CPPD$_{7d}$ and DPPD$_{7d}$ using nontargeted

168 analysis, demonstrating the low reactivity of CPPD and DPPD in this heterogeneous phase

169 condition. Collectively, our study demonstrates the highly structure-related ozone reactivity of

170 PPDs. PPDs with alkyl side chains (*e.g.,* IPPD) appear, based on their ozone reactivity, to be more

171 suitable alternative antioxidants to reduce tire aging.

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173 ***In vitro*** **toxicity of ozonolysis products of PPDs in the coho salmon CSE-119 cell line.** The

174 coho salmon CSE-119 cell line has been reported to be sensitive to 6PPD-Q (21), highlighting its

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175   suitability as an *in vitro* model for the toxicity testing of PPD reaction mixtures. The extracts of

176   individual PPD ozonolysis mixtures were dosed to the CSE-119 cell line at various concentrations

177   (0.686-500 µg/L). The cell viability following 48 hours of exposure was measured using an

178   alamarBlue assay. As shown in Figure 3A, toxicity was not observed for any of the four PPDs at

179   day 0 prior to ozonolysis (PPD_{0d}) as their fluorescence remained unchanged compared with the

180   control groups. Strikingly, a significant decrease in cell viability was observed for both $6PPD_{3d}$ and

181    $6PPD_{7d}$ in a concentration-dependent manner. For example, a cell viability of 41.1 ± 3.3% was

182   observed at 500 µg/L of $6PPD_{3d}$ (Fig. 3B), and an even more pronounced decrease in cell viability

183   (32.5 ± 3.8%) was observed for $6PPD_{7d}$ at the same exposure level (Fig. 3C). The median effective

184   concentration (EC_{50}) of $6PPD_{7d}$ was calculated to be 213.1 µg/L, which was lower than that of

185    $6PPD_{3d}$ (EC_{50} = 401.2 µg/L). This clearly demonstrated the increasing toxicity of the 6PPD reaction

186   mixtures with aging. The concentration of 6PPD-Q in the ozonolysis products of $6PPD_{7d}$ at the EC_{50}

187   (213.1 µg/L) was approximately 17.9 µg/L, which was comparable to the EC_{50} (19.3 µg/L) of pure

188   6PPD-Q measured under the same exposure conditions (Fig. 3E). These results suggest 6PPD-Q

189   as the primary driver of toxicity in the 6PPD ozonolysis mixture and impacts on the viability of the

190   coho salmon cell line.

191   Interestingly, cytotoxicity was not observed for ozonated CPPD or DPPD, while $IPPD_{7d}$ exerted

192   minor cytotoxicity (<10%) but only at the highest concentration (500 µg/L). The limited cytotoxicity

193   of CPPD or DPPD might be due to their low ozone heterogeneous reactivity as aforementioned.

194   Supporting this, no significant cytotoxicity was observed for the parent CPPD or DPPD under the

195   same exposure conditions (Fig. 3D). The low cytotoxicity of the IPPD reaction mixture was

196   surprising, as many transformation products were also formed from IPPD through similar

197   ozonolysis pathways as 6PPD. To investigate the potential mechanism, we tested the cytotoxicity

198   of pure IPPD-Q, which we synthesized (18). In contrast to the strong cytotoxicity of 6PPD-Q, toxicity

199   was not observed for IPPD-Q even at the highest concentration of 100 µg/L (Fig. 3E) (22). This

200   demonstrated that the low toxicity of the IPPD reaction mixture is attributable to the low intrinsic

201   toxicity of IPPD-Q compared to 6PPD-Q. This was consistent with our previous study wherein acute

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202 toxicity in juvenile rainbow trout was only observed for 6PPD-Q amongst seven other tested PPD-

203 Qs including IPPD-Q (18). The results from the coho salmon CSE-119 cell line experiments

204 provided important information: 1) The unique toxicity of 6PPD-Q was observed in both juvenile

205 rainbow trout and coho salmon cells, indicating a common toxic mode of action between the two

206 fish species; 2) Structure-related cytotoxicity was observed in CSE-119, which excluded the

207 impacts of kinetics (*e.g.,* bioaccumulation), and should be attributed to the intrinsic toxicity of 6PPD-

208 Q; 3) Toxicity was not observed for the reaction mixture of IPPD, despite the formation of many

209 transformation products. This demonstrates that other PPD compounds (*e.g.,* IPPD) are potential

210 substitute antioxidants to replace 6PPD.

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212 **96-hour acute toxicity testing of ozonolysis products of PPDs in rainbow trout.** Encouraged

213 by the *in vitro* toxicity results, we tested the *in vivo* toxicity of the ozonolysis mixtures of four PPDs

214 towards juvenile rainbow trout. Rainbow trout is a commonly used animal model for routine

215 chemical, effluent and surface toxicity testing. Additionally, rainbow trout is a salmonid fish species

216 that has demonstrated a high sensitivity to 6PPD-Q toxicity, even at early life stages (9, 18). The

217 fish exposure experiment followed the standardized test method 'Biological Test Method: acute

218 lethality test using rainbow trout' (Report EPS1/RM/9) from Environment and Climate Change

219 Canada (ECCC 1990) (23). The extracts of reaction mixtures of 6PPD were directly dosed to

220 rainbow trout via waterborne exposure at two different nominal concentrations (5.0 and 25 µg/L).

221 These exposure concentrations were selected for two reasons: 1) The concentrations of 6PPD-Q

222 in the reaction mixture were 0.42 and 2.1 µg/L from the 5.0 and 25 µg/L 6PPD$_{7d}$ exposure groups,

223 respectively, which was comparable to the LD$_{50}$ (0.64 µg/L) of pure 6PPD-Q in rainbow trout under

224 comparable exposure conditions (18). 2) The concentrations of 6PPD in surface waters are typically

225 lower than 1.0 µg/L (15, 17), and thus PPDs may be considered 'safe' at environmentally relevant

226 concentration if toxicity is not observed at 25 µg/L.

227 Significant acute toxicity was observed in juvenile rainbow trout exposed to 25 µg/L of 6PPD$_{7d}$ (Fig.

228 4A), the survival rates of rainbow trout continuously decreased with exposure time, which were

229 43.0 ± 20.5%, 20.0 ± 8.2%, 10.0 ± 0% and 10.0 ± 0% after 24, 48, 72, and 96 hr, respectively. The

230 survival rate of rainbow trout after exposure to 25 µg/L of $6PPD_{7d}$ (2.10 µg/L 6PPD-Q) was about

231 43%, which was comparable to the 24 h LC_{50} (2.08 µg/L) of pure 6PPD-Q in our previous study

232 (15). Acute toxicity was also observed for rainbow trout exposed to 25 µg/L of $6PPD_{3d}$, albeit with

233 lesser toxicity (*e.g.,* 53.0 ± 12.5% survival rate at 96 hr) than $6PPD_{7d}$. Toxicity was also observed

234 at 5.0 µg/L of $6PPD_{7d}$ group, but with a higher survival rate of 96.0 ± 4.7% after 96 hr of exposure.

235 In contrast to oxidized 6PPD, toxicity in rainbow trout was not observed for $6PPD_{0d}$ prior to

236 ozonolysis. This demonstrated that the toxicity of reaction mixtures of 6PPD increased along with

237 aging time, which aligned with the results obtained from the *in vitro* coho salmon cell exposures.

238 Interestingly, acute toxicity was not observed in rainbow trout exposed to the reaction mixtures of

239 the three other PPDs (Fig. 3B-D), despite their very similar structures to 6PPD. Although one fish

240 was found dead for one of the three replicates of the 5.0 µg/L of $CPPD_{3d}$ exposure, this should not

241 be attributed to the intrinsic toxicity of CPPD, as toxicity was not observed at higher exposure

242 concentrations (25 µg/L) or at longer exposure times. Collectively, for the first time, we have

243 demonstrated the structurally selective toxicity of ozonated 6PPD, which contrasted with IPPD,

244 CPPD and DPPD, under environmentally relevant ozonolysis conditions. Up to 25 µg/L exposure

245 concentrations were used in the current study which were well above the concentrations of 6PPD

246 in surface waters (15-17). This demonstrated that IPPD may not cause significant acute toxicity

247 towards rainbow trout if used as replacement antioxidants under similar production volumes.

248 However, the toxicity of CPPD and DPPD transformation products remains unclear, as neither

249 appeared to react with ozone under the experimental conditions assessed.

250

251 **Bioaccumulation of PPDs and their transformation products in rainbow trout.** Fourteen of 22

252 transformation products, and 14 of 16 transformation products were detected in the whole fish body

253 for 6PPD and IPPD after the exposure experiments, respectively (Fig. 5, S7-12). Eight of the

254 transformation products were detected in both IPPD and 6PPD treatment groups, including PPD-

255 Qs. The concentrations of 6PPD-Q in the fish body increased with ozonolysis time, with the highest

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256 concentrations detected in the fish body exposed to $6PPD_{7d}$ (Fig. 5B, S7B, S8). 6PPD-Q was

257 detected in a dose-dependent manner, at 2.01 ± 0.44 and 16.8 ± 5.72 ng/g ww in the fish body

258 exposed to 5.0 and 25 μg/L of $6PPD_{7d}$, respectively. These results confirm the bioaccumulation of

259 6PPD-Q in fish following exposure to water dosed with the 6PPD ozonolysis mixtures. IPPD-Q was

260 also detected in the fish body exposed to IPPD reaction mixtures, at slightly lower (2-5 times)

261 concentrations than 6PPD-Q. CPPD-Q was also detected in the fish body, which might be due to

262 the impurity of CPPD-Q in CPPD standard, as we also detected CPPD-Q with very low percentage

263 (~0.3%) in the ozonolysis mixture of $CPPD_{0d}$. Unexpectedly, DPPD-Q was detected in fish exposed

264 to the reaction mixtures of DPPD; it was not detected as a product in the ozonolysis reaction

265 mixtures, as mentioned above. This might be attributed to the oxidation of DPPD by enzymatic or

266 aerobic means, since DPPD-Q was also detected from $DPPD_{0d}$ prior to ozonolysis at similar

267 concentrations. Taken these results together, PPD-Qs were detected in fish body at concentrations

268 of 0.61 to 16.8 ng/g ww after exposure to the reaction mixtures of PPDs (Fig. 5B, S7B). The toxicity

269 of $6PPD_{7d}$ towards both coho salmon cells and rainbow trout fish (18) was similar to that of pure

270 6PPD-Q at the same concentration. From these two pieces of evidence we conclude that 6PPD-Q

271 is the major driver of the selective toxicity of the ozonolysis products of 6PPD.

272 The co-occurrence of 6PPD-Q and its structurally similar transformation products in the same

273 reaction mixture provided an opportunity to identify the key toxicity moieties. For example, high

274 concentrations of 4-HDPA were detected in fish body exposed to all PPDs, except for the DPPD

275 exposure group, at even higher concentrations than their parent PPDs (Fig. 5D, S7D). Note that

276 the concentrations of 4-HDPA were lower than those of 4-ADPA and PPD-Qs in the ozonolysis

277 mixtures of 6PPD and IPPD (Fig. S3A-B). Thus, the high concentrations of 4-HDPA were probably

278 due to the hydrolysis of PPDs via biotic or abiotic processes. The concentrations of 4-HDPA from

279 both the IPPD and CPPD treatment groups were much higher than those of 6PPD, which clearly

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280 demonstrated that 4-HDPA does not cause acute toxicity in rainbow trout, despite its high

281 concentrations.

282 Not surprisingly, parent PPDs were detected in the fish body at relatively high concentrations (Fig.

283 5A, S7A). DPPD, in particular, was detected at greater than 10-times higher concentrations in

284 rainbow trout compared to the three alkyl-PPDs (Fig. 5A). This should not be attributed to

285 hydrophobicity as the $logK_{OW}$ of DPPD (4.04, calculated by EPI Suite) is comparable to or lower

286 than those of 6PPD (4.68), IPPD (3.28) and CPPD (4.64). Instead, slow hydrolysis of DPPD to 4-

287 HDPA might partially explain its high bioaccumulation, considering that 4-HDPA was detected at

288 low concentrations in the DPPD treatment groups. However, toxicity was not observed at day 0 for

289 any PPDs prior to ozonolysis, indicating that parent PPDs are unlikely to be acutely lethal despite

290 10-100 times higher concentrations than 6PPD-Q.

291 Collectively, the results demonstrated that 6PPD-Q drives the selective toxicity of the 6PPD

292 reaction mixture. While a variety of ozonolysis products were detected in fish bodies, our data

293 indicate that other products did not contribute to acute toxicity of the reaction mixture. This

294 demonstrates the unique intrinsic toxicity of 6PPD-Q, and that minor structural modifications on the

295 middle quinone ring or side alkyl chain can completely remove its acute toxicity (**Scheme 1**).

296

297 **Compound- and regio-selective hydroxylation of 6PPD-Q.** Intrigued by the selective toxicity of

298 6PPD-Q, we decided to investigate the structure-related metabolism of 6PPD-Q and other

299 ozonolysis products. More than 11,000 LC-MS features were detected, among them 109 features

300 with >10-time higher signal intensity ($p < 0.05$) from 25 μg/L of $6PPD_{7d}$ compared to $6PPD_{0d}$

301 (negative controls). We focused on high-abundance features with signal intensity greater than

302 1×10^6. Surprisingly, only three LC-MS features were identified as tentative metabolites in positive

303 mode, including m/z = 315.1699 (RT = 5.89 min), m/z = 315.1702 (RT = 6.36 min), and m/z =

304 331.1649 (RT= 5.18 min) (Fig. 6A). The two features at m/z = 315.1699 and m/z = 315.1702, were

305 assigned as the alkyl and aryl hydroxylated metabolites of 6PPD-Q, i.e. 6PPD-Q-OH (alkyl-OH)

306 and 6PPD-Q-OH (aryl-OH) ($C_{18}H_{22}N_2O_3$), respectively. Their identities were clearly supported by

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307   the detection of multiple diagnostic MS^2 fragments (Fig. S13 A and B); the detection of regio-

308   selective 6PPD-Q-OH isomers was consistent with our previous study (18). The feature at m/z =

309   331.1649 (RT = 5.18 min) was assigned as $C_{18}H_{22}N_2O_4$ with a mass error of 0.91 ppm. The MS^2

310   spectrum indicated this feature as di-hydroxylated 6PPD-Q (6PPD-Q-Di-OH), with hydroxylation on

311   both the aromatic ring and alkyl side chain (Fig. S13C). This is the first report of the formation of a

312   di-hydroxylated metabolite of 6PPD-Q in rainbow trout. Due to the lack of authentic standards, we

313   used 6PPD-Q to semi-quantify 6PPD-Q-OH isomers and 6PPD-Q-Di-OH. The total concentrations

314   of the hydroxylated 6PPD-Qs were 2 to 5 times higher than those of 6PPD-Q (Fig. 6B) (18). These

315   results demonstrate the rapid hydroxylation and di-hydroxylation of 6PPD-Q in rainbow trout.

316   Surprisingly, despite the presence of >20 ozonolysis products of 6PPD, metabolites in the fish body

317   were not detected for any of these products through nontargeted analysis. To verify this, we

318   manually inspected for the hydroxylated metabolites of 6PPD and the other ozonolysis products by

319   using their predicted chemical formulae ([M+O]). Hydroxylated metabolites were only detected for

320   6PPD at ~10 times relative abundance lower than 6PPD-Q-OH, despite the 10 to 100 times higher

321   body concentration of 6PPD compared to 6PPD-Q. This was surprising as 6PPD is notably more

322   electron-rich than 6PPD-Q, and thus is predicted to be more prone to undergo oxidative

323   hydroxylation. This demonstrated that the compound-selective hydroxylation of 6PPD-Q should be

324   attributed to the high substrate selectivity of the related enzyme, rather than its chemical reactivity.

325   We then attempted to identify the metabolites of the ozonolysis products of IPPD. Two LC-MS

326   features were detected as tentative metabolites at m/z = 273.1231 (ESI$^+$) and m/z = 351.0654 (ESI$^-$

327   ) (Fig. S14A-B). By collecting MS^1 and MS^2 spectra (Fig. S14C), these two metabolites were

328   assigned as IPPD-Q-OH (aryl-OH) and sulfated IPPD-Q, respectively. The metabolism of IPPD-Q

329   was clearly distinct from 6PPD-Q, in which no alkyl-hydroxylated metabolites were detected (Fig.

330   S14D). Similarly, nontargeted analysis was employed to detect the metabolites in rainbow trout

331   after exposure to ozonolysis mixtures of CPPD and DPPD. While hydroxylated metabolites of

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CPPD were detected at minor levels which were 10-100 times lower than those of parent CPPD, no hydroxylated products were detected from the DPPD group (Fig. S15).

Overall, we herein discovered the highly compound- and regio-selective hydroxylation of 6PPD-Q in rainbow trout: 1) 6PPD-Q showed rapid compound-selective hydroxylation compared to 6PPD and 4-HDPA; 2) 6PPD-Q showed regio-selective hydroxylation on the alkyl side chain, in contrast to IPPD-Q (Fig. 6C). Future studies are warranted to confirm if the regio- and compound-selective hydroxylation of 6PPD-Q contributes to the selective toxicity of the ozonolysis products of 6PPD.

Conclusions. The extremely selective toxicity of the 6PPD ozonolysis mixtures was surprising given the very similar structure of 6PPD relative to the other three PPDs. All evidence indicates that the selective toxicity of the 6PPD ozonolysis-reaction mixture arises from the unique intrinsic toxicity of 6PPD-Q. Notably, the structure-related toxicity of the mixture was observed in both the coho salmon cell line and rainbow trout fish, indicating a common toxic mechanism of action across the two species. While the exact toxicity mechanism remains unknown, the unique compound- and regio-selective hydroxylation of 6PPD-Q indicates that enzymatic bioactivation of the compound may partially contribute to its toxicity. Future studies are warranted to thoroughly investigate the potential role of hydroxylated metabolites and to elucidate the exact toxicity mechanism.

Identifying substitute antioxidants for the replacement of 6PPD is an urgent need. While our recent study pinpointed the potential to use of other PPDs as safer 6PPD substitutes, only PPD-Qs were tested for toxicity (18). The present study is the first to systematically evaluate the toxicity of the ozonolysis products of 6PPD under environmentally relevant oxidation conditions. Importantly, toxicity was not observed for the IPPD ozonolysis mixture, despite the formation of many transformation products that were similar in structure to 6PPD-Q. These results are highly encouraging and support the potential to use novel PPDs as substitutes for 6PPD. However, future

356   studies are warranted to further test the toxicity of PPDs ozonolysis in more animal species (e.g.,

357   *in vivo* coho salmon testing).

358

359

360   **Materials and Methods**

361   **Chemicals and Reagents.** The standards used in this study are listed in Table S1. Solvents and

362   reagents used for sample extraction and mobile phases including acetonitrile, methanol, and formic

363   acid were liquid chromatography-mass spectrometry (LC-MS) grade and purchased from Thermo

364   Fisher Scientific (Waltham, MA, United States). Anhydrous sodium sulfate (Na_2SO_4, purity of >

365   99%), Resazurin sodium salt, and Dulbecco's Phosphate Buffered Saline were bought from Sigma-

366   Aldrich (Oakville, ON, Canada). Sodium Chloride (NaCl, purity of > 99.5%) was purchased from

367   Bioshop (Burlington, ON, Canada). C18 was purchased from Waters (Mississauga, ON, Canada).

368   Reagents and media used for the cell cultures, such as Penicillin-Streptomycin (10,000 U/mL),

369   Gibco Fetal Bovine Serum, Leibovitz's (1X) (L-15 Medium), 0.25% Trypsin-EDTA (1X), were also

370   purchased from Thermo Fisher Scientific (Waltham, MA, United States).

371

372   **Ozonolysis of PPD Surface Film in a Flow Reactor.** The ozonolysis experiment was conducted

373   in a cylindrical quartz flow reactor with Teflon flanges. The reactor was placed in a metall box to

374   block natural light from the room. The ozonolysis experiments were carried out at room temperature

375   (~295 K), and relative humidity (RH) was maintained at 45-50% inside the flow reactor. The

376   experimental conditions and setup were adapted from our previous study (24). To simulate an

377   atmospherically-relevant ozone condition, the ozone mixing ratio in the flow reactor was maintained

378   at 100 ppb. A total of 2.0 L/min airflow was sent to the flow reactor: specifically, 10 cubic centimeter

379   per minute (ccm) zero air passed through an ozone generator that was composed of a quartz cell

380   and a Pen−Ray lamp, 1.0 L/min humidified zero air flow passed through a bubbler filled with distilled

381   water, and 1.0 L/min dry zero air flowed directly to the flow reactor. At the outlet flow, the ozone

382   mixing ratio and RH were monitored by an UV photometric ozone analyzer (Thermo Scientific,

383   Model 49C, USA) and a hygrometer (Vaisala, USA), respectively.

384   The ozonolysis of select PPDs (6PPD, IPPD, CPPD, and DPPD) was performed individually in the

385   form of surface films. Each PPD film sample was deposited on a circular cover glass slide (25 mm

386   diameter, Fisher Scientific) by covering the glass slide with a 130 µL aliquot of 5.0 mg/mL PPD in

387   acetone and waiting for full solvent evaporation in ambient air (~3 minutes). The PPD film samples

388   were then transferred to the flow reactor for ozone exposure (100 ppb) for 3 and 7 days to simulate

389   different atmospheric ozone exposure scenarios. Triplicates were prepared for each time point.

390   Then, each film sample was extracted with 2.6 mL HPLC-grade acetonitrile (250 mg/L of PPD_{0d-7d}

391   mixtures), 0.5 mL of which was further diluted to a final concentration of 50 mg/L with acetonitrile.

392   0-day film samples were extracted immediately after solvent evaporation with minimal ambient

393   ozone exposure (< 5 min). Zero air control samples for each PPD were also collected under the

394   same exposure periods and flow conditions, except for turning the Pen-Ray lamp on for ozone

395   supply. The ozone mixing ratio was below 2 ppb during control experiments. The extracted

396   solutions were stored at -80 °C until LC-HRMS analysis.

397

398   **Fish Source and Culture.** The rainbow trout experiments were conducted in the Ontario Ministry

399   of the Environment, Conservation and Parks (MECP, Scarborough ON, Canada) Laboratory

400   Services Branch. Eggs of rainbow trout were purchased from Lyndon Fish Hatcheries (New

401   Dundee, ON, Canada). The culturing of rainbow trout was the same as outlined in our previous

402   study (18). After being hatched from eggs, fish were cultured under flow-through conditions at 15

403   ± 1 °C for six weeks before exposure experiments. Daily monitoring and feeding with a commercial

404   fish feed at a daily rate of 1% of body weight were administered.

405

406   **96 hr Acute Toxicity Test in Rainbow Trout.** The 96-hour acute toxicity of PPDs ozonolysis

407   mixtures was tested by using 1.5 – 3 month-old juvenile rainbow trout (0.2 – 0.4 g). Fish exposure

408   procedures strictly followed the 'Biological Test Method: acute lethality test using rainbow trout'

409 (Report EPS1/RM/9) from Method Development and Applications Section, Environmental

410 Technology Centre, Environment and Climate Change Canada (ECCC 1990) (23). Organism

411 health, technician proficiency and environmental conditions were confirmed before the exposure

412 experiment and monthly during the experimental process using reference tests with potassium

413 chloride (KCl) (ECCC 1990). All experimental protocols were approved by the University of Toronto

414 Animal Care and Use Committee.

415 Exposures of rainbow trout were conducted in buckets lined with food-grade polyethylene

416 disposable liners, which contained 20 L dechlorinated water and 10 rainbow trout, maintained at

417 15 ± 1 °C in a temperature-controlled room. The exposure media was continuously aerated through

418 compressed air bubbled through air stones directly into each vessel to maintain consistent

419 dissolved oxygen (DO) levels between replicates. Fluorescent lighting was maintained between

420 100-500 lux on a 16:8 Light:Dark cycle. For each PPD group, two concentrations (5.0 and 25 µg/L)

421 of oxidized PPD at three ozonolysis time points (0 d, 3 d, 7 d) were tested, by adding 2 mL of

422 ozonolysis mixture of PPD (250 mg/L and 50 mg/L) into 20 L dechlorinated water. For each

423 exposure concentration and each ozonation time point, three replicates were performed. The

424 number of fish mortalities was recorded and temperature was confirmed daily. During the 96 hr

425 exposure experiments, the dead fish were taken out from the exposure containers every 24 hr and

426 preserved at -80 °C. After 96 hr, the fish surviving from the exposure and control groups were

427 euthanized using CO_2 in water and kept at -80 °C until analysis.

428

429 **Cell Viability Assay.** Cellular metabolic activity was tested to determine viability using alamarBlue

430 cell viability reagent (21), which was prepared by dissolving Resazurin sodium salt in phosphate

431 buffered saline at a concentration of 0.30 mg/mL, which was passed through a sterilizing filter (0.22

432 µm) and stored at -20 °C in darkness. One day before exposure, CSE-119 cells were seeded in a

433 96-well plate with a density of 10,000 cells/well in 100 µL of culture media. Exposure media was

434 prepared with PPDs ozonolysis mixtures dosed in fresh culture media. Then, media in 96-well

435 plates were replaced with 100 µL of seven 3-fold serial diluted exposure media (0.686 to 500 µg/L).

18

436   The percentage of acetonitrile in the culture media was less than 0.2%, and acetonitrile at this

437   percentage did not influence cell viability. There were 12 replicates for each concentration. After

438   48 hours at 20 °C, 10 µL of alamarBlue cell viability reagent was added to each well in a dark room,

439   then the cells were incubated for 4 h at 20°C in darkness. After that, fluorescence was recorded

440   with excitation at 550 nm and emission at 600 nm by a Tecan Infinite 200 Pro M Plex Microplate

441   Reader (Tecan Life Sciences, Switzerland). Cell viability was calculated as the measured

442   fluorescence value at each well compared with that of the solvent control from the same plate. Cell

443   viability of pure PPDs and PPD-Qs was also tested by the same method.

444

445   **Sample Preparation.**

446   *PPD/O$_3$ mixtures.* After extraction by acetonitrile, the ozonolysis mixtures of PPD were diluted until

447   the concentrations of PPD$_{0d}$, PPD$_{3d}$, and PPD$_{7d}$ reached 100 µg/L, and 6PPD-Q-d$_5$ was added at

448   a final concentration of 10 µg/L. Subsequently, they were subjected to LC-HRMS analysis.

449   *Fish samples.* The pretreatment method of fish samples was similar to our previous study with

450   minor modification (18). Briefly, 300 mg of NaCl was weighed into a 1.5 mL Eppendorf tube, and a

451   whole fish was transferred to the tube after thawing. The wet weight of fish was recorded. The fish

452   body was homogenized (n = 3), and 12 ng of 6PPD-Q-d$_5$ was added (for the recovery experiment

453   for method validation, 24 ng of standard mixtures were added simultaneously). Following a 1 hr

454   equilibration period, 300 µL of H$_2$O and 600 µL of acetonitrile were introduced, and the samples

455   were vortexed for 1 min. Each sample underwent 20 min of shaking, 20 min of sonication, and

456   subsequently was centrifuged for 5 min at 10,000 x *g*. After delicate transfer of the supernatant to

457   a new 1.5 mL tube, another 600 µL of acetonitrile was used to repeat the extraction. The

458   supernatants were consolidated, and subsequently, 3.6 mg of C$_{18}$ and 40 mg of Na$_2$SO$_4$ were

459   introduced to remove lipids and dry the organic extracts. Following a 1 min vortex, the combined

460   extracts underwent centrifugation at 10,000 × *g* for 5 min. Then, the supernatant was transferred

461   to a new amber glass vial for LC-HRMS analysis. The recoveries of PPDs and their transformation

19

462   products (n = 15) were satisfactory, which were 75.4 ± 8.9% ~ 126.4 ± 3.1%, meeting the

463   requirements for quantification (Fig. S6).

464

465   **LC-HRMS Analysis**. The samples were measured by a Q Exactive orbitrap mass spectrometer

466   (MS) equipped with a Vanquish ultra-high performance liquid chromatography (UPLC) system

467   (Thermo Fisher Scientific, USA). Chemical separation was carried out using a C_{18} column (50 mm

468   × 2.1 mm, 1.5 μm), which was obtained from Thermo Fisher Scientific, USA, and 2 μL of each

469   sample was injected. The temperature of the auto-sampler was kept at 4 °C, while the temperature

470   of the column chamber was held at 40 °C. The mobile phases were composed of 0.1% formic acid

471   in water (A) and 0.1% formic acid in methanol (B). The gradient elution was performed as follows:

472   0 – 1.5 min, 5.0% B; 3.5 min, 50% B; 7.5 – 11.0 min, 100% B; 11.2 – 12.0 min, 5.0% B, and

473   operated at a flow rate of 0.25 mL/min.

474   The scan mode involved a combination of a full MS^1 scan followed by subsequent data-independent

475   acquisition (DIA) scans. The full MS^1 scan covered the m/z range of 100 to 900 at a resolution (R)

476   of 70,000 (at $m/z = 200$), facilitating the collection of a maximum of 1×10^6 ions within 60 ms. In

477   both negative and positive modes, two DIA tandem MS (MS/MS) scans were established by

478   covering the m/z ranges of 100 to 500 and 500 to 900, respectively. The DIA scan had an isolation

479   window of 20 m/z; other parameters included a resolution (R) of 35,000 (at $m/z = 200$), an AGC

480   target of 1×10^5, and a maximum ionization time of 50 ms. The parameters specified in the tune file

481   were as follows: a spray voltage of 2.8 kV, a sheath gas flow rate of 30 L/hr, an auxiliary gas flow

482   rate of 7 L/hr, and a capillary temperature of 300 °C.

483

484   **Nontargeted Analysis.** Nontargeted analysis was performed using our previously developed in-

485   house algorithm (25, 26). The first step involved transforming the raw mass spectrometry files into

486   an ".mzXML" format; after this, the features in the data files were identified after running an R

487   package (27). To investigate the transformation products in ozonolysis mixtures of PPDs, the

488   features in each PPD_{7d} were contrasted with those found in PPD_{0d}. Those features with significantly

20

489 ($p < 0.05$) higher (fold change > 5) signal intensity were selected and further analyzed. Likewise,

490 the features identified in fish body were also examined. Features with signal intensity higher than

491 1×10^6 and those exhibiting 10-fold significantly higher ($p < 0.05$) signal intensity in 25 µg/L of PPD_{7d}

492 in comparison with those in fish body after exposure to 25 µg/L of PPD_{0d} were chosen for analysis,

493 as the abundant metabolites suggested a substantial role in both toxicity and bioaccumulation. The

494 chemical formulae were predicted using the *Xcalibur Qual Browser* software (Thermofisher

495 Software Version 4.1) after inputting the *m/z* of the feature within the mass tolerance of 5 ppm and

496 specifying the type and number isotopes (^{12}C: 6 – 40; ^{1}H: 5 – 50; ^{16}O: 0 – 10; ^{14}N: 0 – 10; ^{32}S: 0 –

497 4). The chemical structures of the identified peaks were proposed by three methods as follows: 1)

498 For those chemicals with commercially available standards, the structures of the identified features

499 were confirmed by comparing the retention times and corresponding MS/MS fragments with

500 purchased standards; 2) For chemicals with the same formulae as compounds from the published

501 literatures, their structures were proposed by comparing their retention times on a C_{18} column

502 relative to PPD and PPD-Q, along with MS^2 fragments reported in previous studies; 3) For other

503 identified peaks, chemical structures were hypothesized by analyzing the MS^2 fragments. The

504 concentrations of transformation products from the $6PPD_{0d-7d}$ and the metabolites from fish after

505 exposure to $6PPD_{0d-7d}$ were semi-quantified by using the standards of 6PPD-Q and 6PPD-Q-d_5.

506 The concentrations of transformation products from the $IPPD_{0d-7d}$ and the metabolites from fish

507 after exposure to $IPPD_{0d-7d}$ were semi-quantified by using the standards of IPPD-Q and 6PPD-Q-

508 d_5.

509

510 **Quality Control and Quality Assurance.** During the ozonolysis process, zero air control samples

511 were also included by using the same chamber with the O_3 concentration held lower than 2 ppb, to

512 confirm that the transformation of PPDs was induced by O_3. A solvent control exposure group (n =

513 3) was included into the rainbow trout exposure experiment, wherein 2 mL of pure acetonitrile was

514 spiked. Additionally, another control group (n = 3) of rainbow trout exposure to pure dechlorinated

515 water was included, in which the weight and length were determined at the end of the 96-hr

516   exposure. It was confirmed that that the size of fish met the standard method criteria (ECCC 1990).

517   In the dechlorinated water and solvent controls for all exposures the survival criteria of $\leqslant 10\%$

518   mortality was met (Figure 4). During the process of fish samples preparation, procedure blanks

519   were also included without fish to monitor for possible background contamination. While processing

520   the LC-HRMS analysis, standard mixtures were injected after every 15 samples to check the

521   stability of the instrument. Methanol was also injected after every 15 samples to assess potential

522   carry over. The calibration curve, ranging from 0.1 to 100 ng/mL, showed a high linear regression

523   coefficient ($R^2 > 0.99$) for each chemical. 6PPD-Q-d$_5$ was used as the internal standard over the

524   course of quantification. The recoveries of PPDs and transformation products were in the range of

525   75.4 ± 8.9% to 126.4 ± 3.1% (Fig.S6). Method detection limits (MDLs) were determined based on

526   the 99% confidence level of the y-intercept divided by the slope of the calibration curve, which was

527   0.10 – 1.0 ng/mL in PPD/O$_3$ mixtures and water samples, and 0.522 – 1.30 ng/g in fish body.

528

529   **Statistical Analyses**. Statistical analyses were carried out primarily using GraphPad Prism

530   (v10.1.2, GraphPad software Inc, Boston, MA, USA) (28) and *R* studio (v4.2.1, RStudio, Inc.,

531   Boston, MA, USA) (29).

532

533

542   reviewed and approved for publication consistent with USGS Fundamental Science Practices

543   (https://pubs.usgs.gov/circ/1367).

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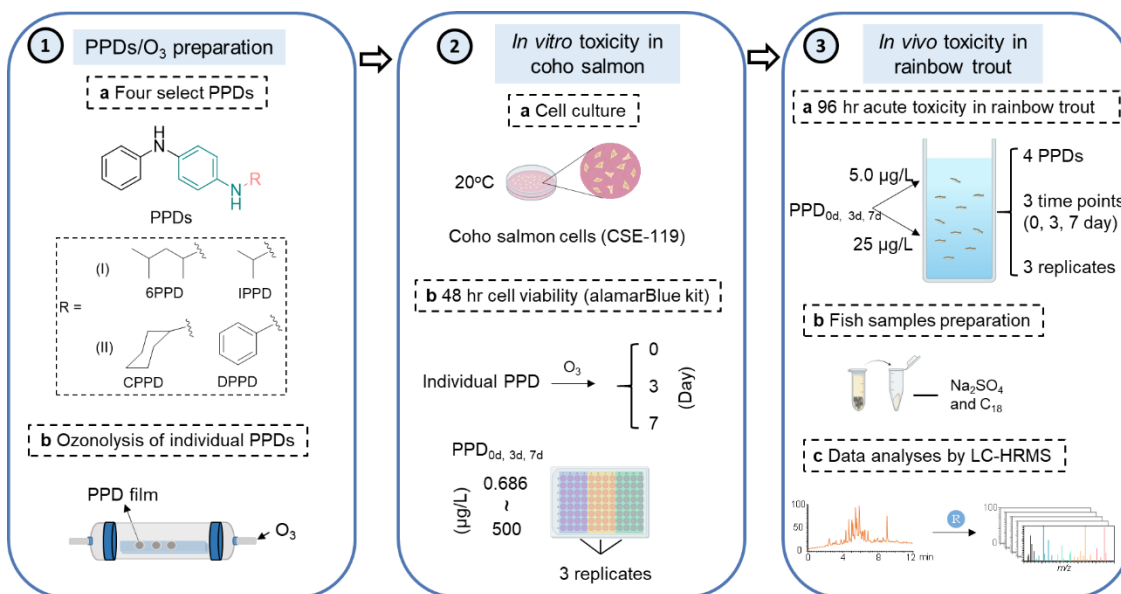
Fig. 1. Workflow for the ozonolysis and toxicity testing of four selected PPDs. 1) Ozonolysis of PPDs in the flow reactor in the presence of 100 ppb of ozone. 2) *In vitro* toxicity of ozonolysis products using coho salmon CSE-119 (*Oncorhynchus kisutch*) cell line. 3) *In vivo* toxicity and biotransformation of ozonolysis products using juvenile rainbow trout (*Oncorhynchus mykiss*).
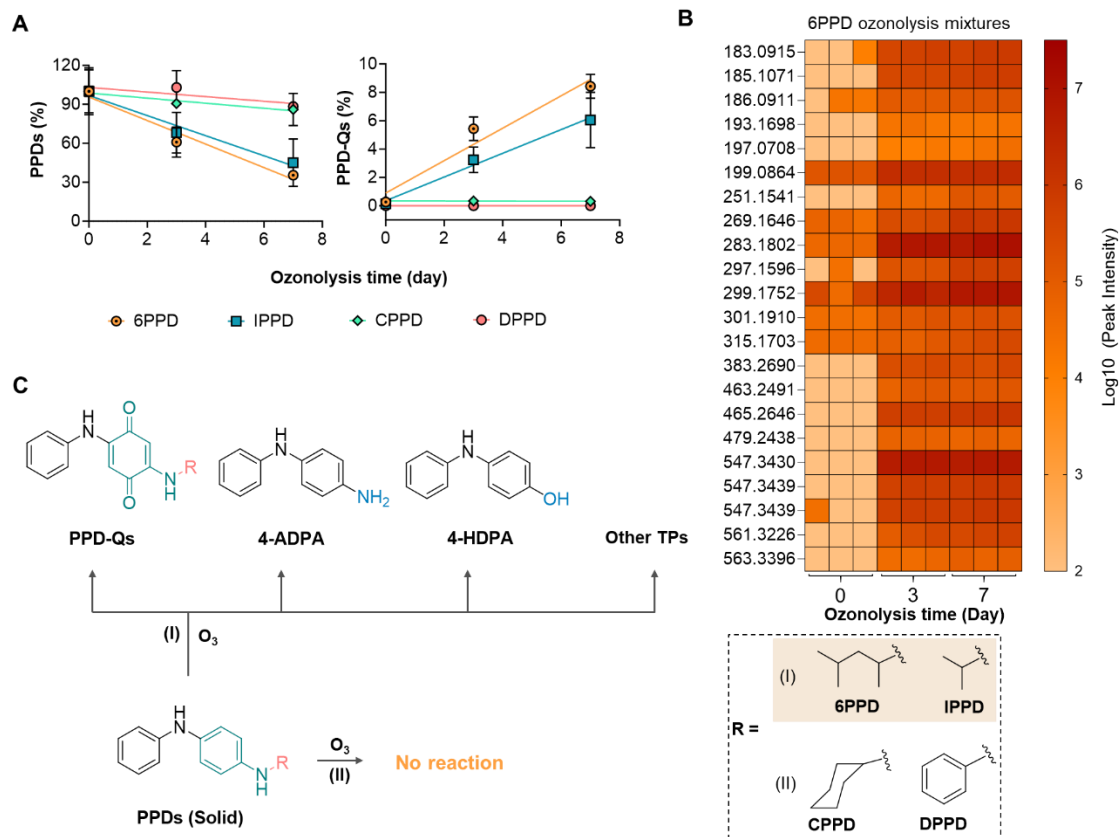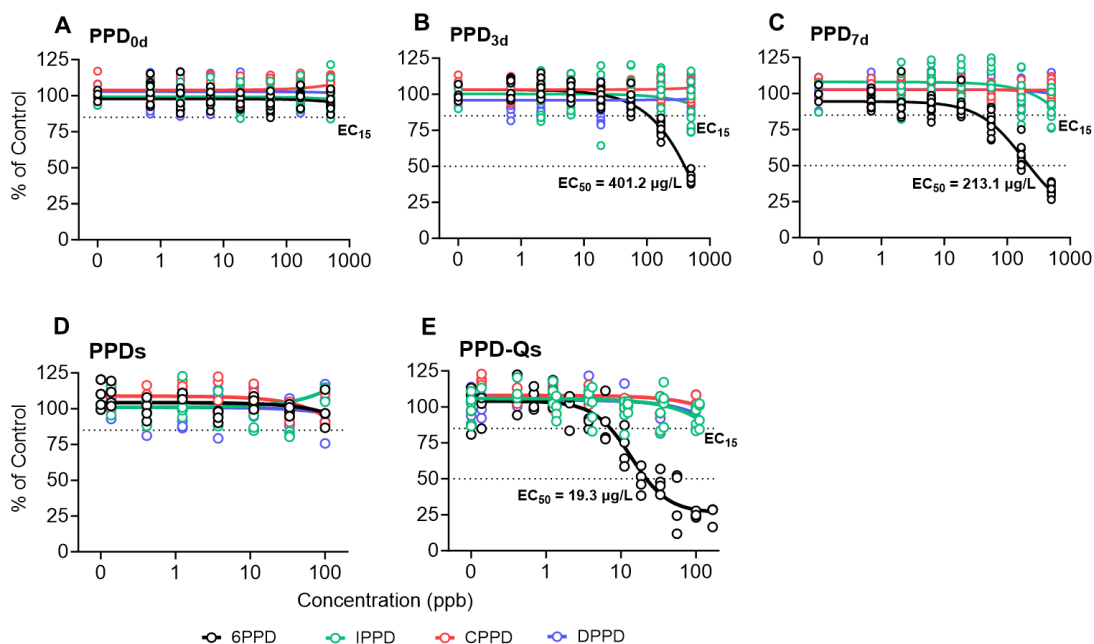
633

634 **Fig. 2.** Ozonolysis of PPDs. (A) The decay of PPDs and growth of PPD-Qs along with aging time

635 (Day 0, 3 and 7). (B) Transformation products of 6PPD after ozonolysis, and their increasing peak

636 abundances along with aging time; (C) Structurally selective reactions of 6PPD and IPPD with

637 ozone via representative reaction pathways.

638

27

639

Fig. 3. *In vitro* cytotoxicity of the ozonolysis products of PPDs in coho salmon (*Oncorhynchus kisutch*) CSE-119 cell line. (A) Toxicity of PPDs on Day 0 prior to ozonolysis; (B) Toxicity of PPDs after 3 days of ozonolysis; (C) Toxicity of PPDs after 7 days of ozonolysis; (D, E) Toxicity of pure PPDs and PPD-Qs. Control groups refer to the cells exposed to the culture medium containing the same volume of organic solvents which was used to dissolve the samples or standards. The two dashed lines indicated the median effective concentration (EC_{50}) and the 15% effective concentration (EC_{15}).
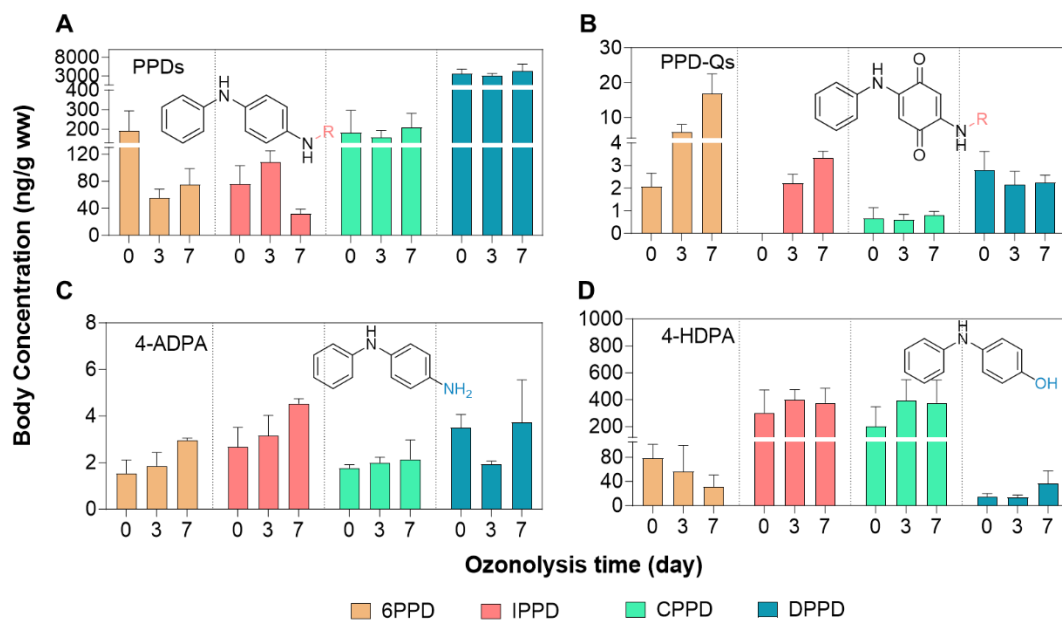
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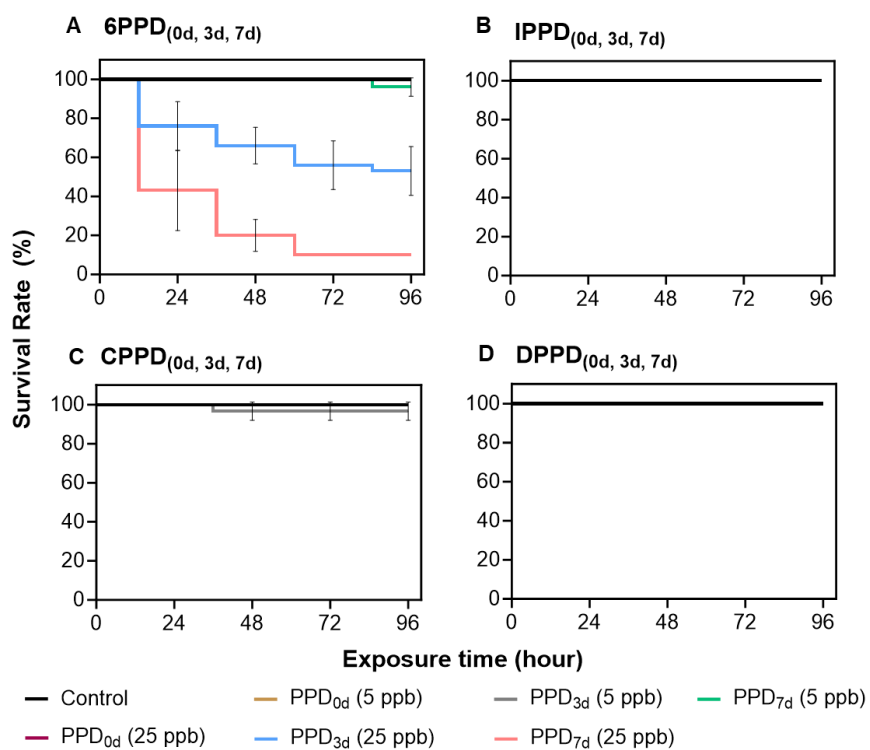
Fig. 4. Acute toxicity of the ozonolysis products of PPDs in rainbow trout (*Oncorhynchus mykiss*).

649   96-hour survival rates of rainbow trout (n=3) after the exposure of 6PPD (A), IPPD (B), CPPD (C),
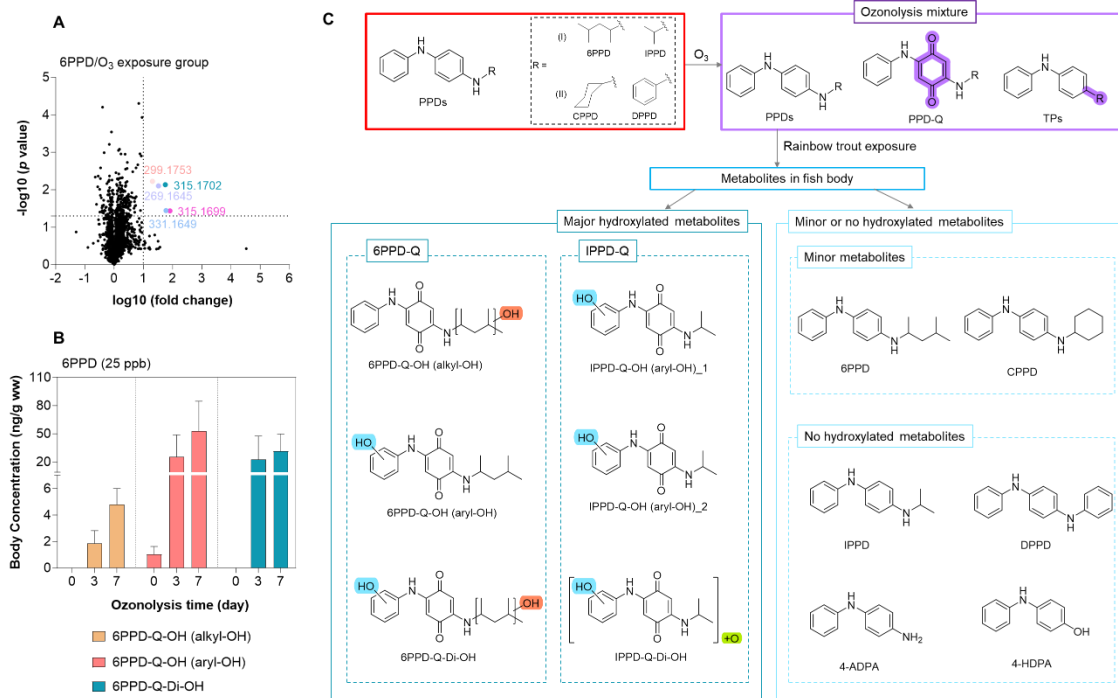
650   and DPPD (D) reaction mixtures.

651

652

Fig. 5. Concentrations of PPDs and representative transformation products in the whole body of

rainbow trout (*Oncorhynchus mykiss*) exposed to 25 µg/L of PPD reaction mixtures.

Concentrations of PPDs (A), PPD-Qs (B), 4-ADPA (C), and 4-HDPA (D).

656

657

Fig. 6. Selective formation of hydroxylated metabolites from 6PPD-Quinone. (A) Nontargeted

detection of metabolites of 6PPD-Quinone from rainbow trout (*Oncorhynchus mykiss*) body; (B)

The concentrations of hydroxylated 6PPD-Q in rainbow trout exposed to 25 µg/L of 6PPD

ozonolysis mixtures; (C) Compound and regio-selective hydroxylation of 6PPD-Q, compared to

other structurally related compounds.

663

664
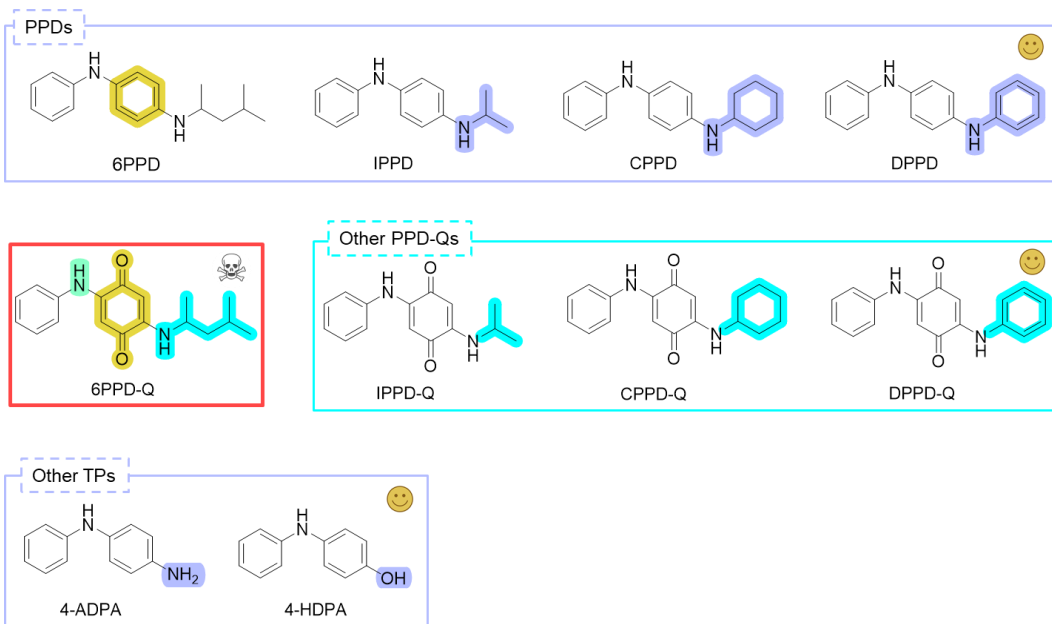
Scheme 1. Structure-related toxicity of 6PPD-Quinone in rainbow trout (*Oncorhynchus mykiss*) relative to other nontoxic ozonolysis products. Key toxicity moieties on 6PPD-Quinone were labeled in yellow or blue.