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Environmental significance (< 120 words)

Since the identification of 6PPD-quinone in late 2020, the assessment and design of alternatives to 6PPD have become urgent, worldwide priorities due to its ubiquity and high acute toxicity of 6PPD-quinone to salmonids. 7PPD-quinone and 77PD-quinone are quinone derivatives of candidate alternatives but currently lack toxicological data in non-salmonid aquatic species. Here, we provide the first toxicity data for early-life stages (embryos and sac-fry) of zebrafish exposed to 7PPD-quinone and 77PD-quinone, revealing sublethal developmental effects at high concentrations ($\geq 500 \mu\text{g/L}$). These findings advance the characterization of ecological risks associated with tire-rubber additives and inform safer alternatives assessment.

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Sublethal toxicity assessments of 7PPD-quinone and 77PD-quinone in zebrafish embryos and sac-fry

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ABSTRACT (limit, 250 words)

High salmonid toxicity of 6PPD-quinone, a transformation product of the tire antioxidant, 6PPD, has accelerated the search for safer tire alternatives. We evaluated the toxicity of the quinone transformation products of two candidate alternatives, 7PPD-quinone (7PPD-Q) and 77PD-quinone (77PD-Q), in zebrafish (*Danio rerio*) early life stages. Embryos and sac-fry were exposed to these chemicals under OECD Test Guideline 212 conditions. Endpoints included 8-day survival, hatching, heartbeat, eye and head sizes, body length, morphological deformities, and yolk-sac pigmentation. Results showed that no 8-day lethality occurred for either chemical up to 2000 $\mu\text{g/L}$. 7PPD-Q produced significant, concentration-related reductions in hatching success (2000 $\mu\text{g/L}$), eye (≥ 1000 $\mu\text{g/L}$) and head sizes (≥ 500 $\mu\text{g/L}$), and yolk-sac pigmentation (≥ 500 $\mu\text{g/L}$). By contrast, 77PD-Q significantly reduced pigmentation only (≥ 1000 $\mu\text{g/L}$). Neither chemical produced concentration-dependent changes in heartbeat, overt malformations, or body length. These results indicate that early-life zebrafish are far less sensitive to these PPD-quinones than coho salmon (*Oncorhynchus kisutch*). Given the high observed effect concentrations, which approached or exceeded the predicted water solubility limits, developmental risk of 7PPD-Q and 77PD-Q to zebrafish appears low.

Keywords: tire rubber additive, p-phenylenediamine, 6PPD-quinone, transformation product

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INTRODUCTION

Tire wear particles (TWPs) are a pervasive non-exhaust traffic emission, carrying a complex mixture of rubber additives into aquatic ecosystems. A recent study revealed that the antioxidant *N*-(1,3-dimethylbutyl)-*N'*-phenyl-*p*-phenylenediamine (6PPD) undergoes rapid ozonation to form 6PPD-quinone (6PPD-Q), which induces acute mortality in coho salmon (*Oncorhynchus kisutch*) at environmentally relevant concentrations^{1,2}. Subsequent studies have detected 6PPD-Q worldwide in various environments, such as urban roads^{3,4}, surface waters⁵, sediment⁶, atmospheric particles⁷, and even in the human body^{8,9}. In addition, 6PPD-Q has been shown to cause acute mortality in several salmonid species, such as lake trout (*Salvelinus namaycush*)¹⁰, brook trout (*S. fontinalis*)¹¹, and white-spotted charr (*S. leucomaenis pluvius*)¹². Interestingly, the acute mortality of 6PPD-Q has not been observed in some other salmonid and non-salmonid species at environmentally realistic concentrations^{11–15}.

The toxicity and ubiquity of 6PPD-Q have triggered regulatory scrutiny and product redesign initiatives¹⁶. In the United States, California has listed motor-vehicle tires containing 6PPD under its Safer Consumer Products program, promoting an industry-led alternatives analysis¹⁷. The U.S. Tire Manufacturers Association (USTMA) released a preliminary assessment that recommended further evaluation of other PPDs (i.e., 7PPD, IPPD, 77PD, CCPD) and non-PPD chemicals (i.e., octyl gallate and Irganox 1520) as potential alternatives to 6PPD¹⁷. Because these PPD candidates possess similar chemical structures to 6PPD, their quinone transformation products may exhibit analogous ecological hazards and therefore warrant hazard screening.

Recent studies have tested the toxicity of these PPDs and their transformation products in salmonid species. No acute lethal toxicity was observed for 77PD-quinone (77PD-Q) in coho salmon up to 226 µg/L, while 77PD was lethally toxic with a 96-h LC₅₀ value of 24 µg/L¹⁸. Nair *et al.* tested the acute toxicity of a suite of PPD-quinones in rainbow trout (*O. mykiss*), revealing that the tested PPD-quinones, including IPPD-quinone (IPPD-Q), CPPD-quinone, and 77PD-Q, did not exhibit acute mortality¹⁹. Similarly, a test report demonstrated the absence of acute lethality in rainbow trout for



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4 ozonation products of both 7PPD and 77PD²⁰. In contrast, 7PPD-quinone (7PPD-Q) showed acute
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6 lethality to juvenile coho salmon with a 24-h LC₅₀ of 5.4 µg/L, whereas 8PPD-quinone (8PPD-Q)
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8 did not induce significant lethality at 61 µg/L²¹. In addition, imaging-based assays using rainbow-
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10 trout gill cells (RTgill-W1) demonstrated that phenotypic effective concentrations for 7PPD-Q and
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12 77PD-Q were comparable to those for 6PPD-Q, whereas IPPD-Q and parent PPDs showed higher
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14 effective concentrations²². Despite these advances, the toxicity of PPDs and PPD-quinones to
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16 non-salmonid aquatic species remains insufficiently characterized, which is essential for
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18 comprehensive ecological risk assessments. In particular, PPD-quinones are emerging contaminants
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20 and lack ecological toxicity data. One notable exception is a study using the bacterium *Vibrio*
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22 *fischeri* to assess toxicity across a range of PPDs and PPD-quinones, including 7PPD-Q, 8PPD-Q,
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24 IPPD-Q, and 77PD-Q²³.

To fill these data gaps, we performed short-term toxicity tests of 7PPD-Q and 77PD-Q using early
life stages of zebrafish (*Danio rerio*) following an OECD test guideline²⁴. We selected 7PPD-Q and
77PD-Q, because their parent chemicals, 7PPD and 77PD, were identified by industry as promising
alternative candidates to 6PPD¹⁷. In addition to standard endpoints described in the test guideline,
we evaluated additional sublethal endpoints such as eye size and pigmentation rate, as these have
been reported to be affected in zebrafish embryos exposed to 6PPD or 6PPD-Q^{25–29}.

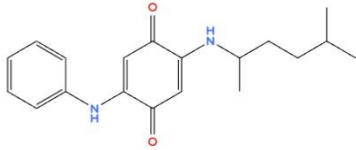
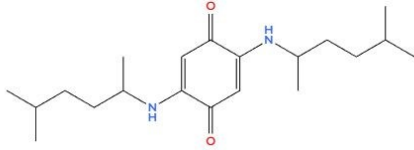
MATERIALS AND METHODS

Chemicals

7PPD-Q (CAS: 2894124-00-4, purity: > 95%) and 77PD-Q (CAS: not available, purity: > 98%)
were purchased from Cayman Chemical. [¹³C₆] 6PPD-Q (>98%) dissolved in acetonitrile was
purchased from Cambridge Isotope Laboratories (USA). The chemical structure and predicted
physico-chemical information of these chemicals are shown in Table 1. Acetone (> 99.5%),
methanol (> 99.8%), and DMSO (> 99.0%) were purchased from Fuji Film Wako Pure Chemical
(Japan).



Table 1. Predicted physico-chemical characteristics and chemical structure of 7PPD-Q and 77PD-Q.

	7PPD-Q	77PD-Q
CAS	2894124-00-4	Not available
Chemical structure		
Molecular weight	312.41	334.50
Aqueous solubility (µg/L)	803	215
log K_{OW}	3.69	4.52
p K_a	11.77	Not available

Aqueous solubility, log K_{OW} , and p K_a were predicted by Marvin³⁰.

Fish source and culture

Zebrafish (*D. rerio*) was obtained from a brood stock at the National Institute for Environmental Studies, Japan (NIES-R strain), which has been maintained for more than 18 years. The brood stock was kept in about 5 L of dechlorinated tap water (hardness; about 80 mg CaCO₃/L) at 26°C under a light-dark cycle of 16:8 h and fed brine shrimp (*Artemia* spp.) on weekdays and dry fish food on weekends. On the evening before starting exposure test, three female zebrafish were transferred to a glass tank containing dechlorinated tap water and glass beads. The following morning, just after the lights were turned on, two male zebrafish were added to the tank. After natural mating for about 1 hour, eggs were collected from the glass beads and examined for fertilization under a stereomicroscope. All the fish used in this study were treated humanely in accordance with the Guideline for the Care and Use of Laboratory Animals of the National Institute for Environmental Studies.

Exposure test

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Lethal and sublethal toxicity tests were performed according to the OECD Test Guideline 212²⁴ with minor modifications. Six nominal 7PPD-Q or 77PD-Q concentrations were set according to a preliminary range-finding experiment (data not shown): 0 (DMSO control), 125, 250, 500, 1000, and 2000 µg/L, each containing ≤ 0.04% (v/v) DMSO as the carrier solvent. In addition, dechlorinated tap water without DMSO was tested as negative control. Test solutions at 2000 µg/L were prepared by adding 80 µL of the 7PPD-Q or 77PD-Q stock solution to 200 mL of dechlorinated tap water, followed by sonication for 15 minutes and stirring for 5 minutes. The resulting solution was then diluted with dechlorinated tap water to obtain the lower test concentrations. Within 90 minutes after fertilization, ten embryos were transferred to each glass beaker containing about 30 mL of the test solution. Three beakers were used for each concentration (n = 3). During the exposure for 8 days, test beakers were kept at 26°C under a light-dark cycle of 16:8 h. No aeration and additional food were provided. Approximately 90% of the test solutions were replaced daily with a freshly prepared solution. Test solution samples were collected for chemical analysis using a glass pipet just after starting exposure (about 2 hpf) and before and after water replacement at 24, 48, 72, and 144 hpf (Table S1). Basic water quality parameters, including temperature, dissolved oxygen, pH, and conductivity, were measured daily before water replacement.

Observation

Test individuals were observed daily under microscope (SZX10, Olympus). Lethality during embryo stages was determined based on the criteria by OECD Test Guideline 236³¹: the embryo was considered dead, if any of the following four endpoints was observed: coagulation, failure of somite formation, non-detachment of the tail, or absence of heartbeat. Larvae and sac-fry were considered dead and removed when completely immobile and no detectable heartbeat. In addition to lethal endpoints, hatching success and abnormal appearance were recorded daily, including edema and body curvature, according to a previous study³².

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4 *Heartbeat rate:* Videos were recorded at 54–56 hpf for ten individuals randomly selected from
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6 each treatment group using a microscope (MVX10, Olympus) equipped with a digital camera (DP80)
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8 and imaging software (cellSens). Heartbeat rate was quantified by first using ImageJ (version 1.54p)
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10 with the Time Series Analyzer plugin to extract a trace of pixel-intensity fluctuations from the
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12 recorded videos ³³, and then manually counting the number of peaks in the pixel-intensity trace for
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14 15 seconds.

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17 *Eye and head sizes:* Images of hatched embryos at 72 hpf were captured using the MVX10
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19 microscope. Fifteen to twenty embryos were randomly selected from each treatment. Eye and head
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21 sizes were quantified using Image J and its polygon selection tool. Head region was defined
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23 according to a previous study ³⁴, as the area enclosed by the tip of the nose, eye centroid, otolith, and
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25 the beginning of the first somite.

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27 *Pigmentation rate:* The images used for eye and head size analysis were also used for the
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29 quantification of pigmentation rate. Yolk-sac region pigmentation was quantified by measuring
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31 grayscale values using ImageJ ²⁶.

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Chemical analysis

Concentrations of 7PPD-Q and 77PD-Q in the test solutions were determined using an LC/MS/MS system (LCMS-8060NX, Shimadzu). The collected water samples were mixed with the same volume of 100 µg/L surrogate [¹³C₆]-6PPD-Q in acetonitrile, diluted with 20 times by the mixture of water and acetonitrile, and then 1 µL aliquots were injected into the LC/MS/MS system with a Shim-pack VP ODS column (150 mm×2.0 mm, silica-based C18stationary phase). The mobile phase was 0.1% ammonium acetate and methanol (1:8 v/v) at an isocratic flow rate of 0.18 mL/min. All chemicals were analyzed in positive electrospray ionization mode and detected by multiple reaction monitoring. The transitions from m/z 313 to 187 ³⁵, m/z 335 to 237 ³⁶, and m/z 305 to 247 ¹² were used for the quantification of 7PPD-Q, 77PD-Q, and [¹³C₆]-6PPD-Q, respectively. Additional transitions (from m/z 305 to 107, m/z 335 to 139) were monitored to confirm the presence

of 7PPD-Q and 77PD-Q. The retention times were 3.8 min (7PPD-Q), 5.0 (77PD-Q), and 3.4 min ([¹³C₆]-6PPD-Q). All analytes were quantified using eight-point calibration curves (5 to 250 µg/L for target chemicals and 5 µg/L for the surrogate).

Statistical analysis

For heartbeat, eye size, head size, pigmentation rate, and body length, statistically significant differences were evaluated using linear mixed-effects models, with treatment as a fixed effect and beaker as a random effect nested within treatment, using the R software ver. 4.4.2³⁷ and the lme4 package ver. 1.1-38³⁸. Post hoc comparisons with the DMSO control were conducted by Dunnett's test using the multcomp package ver. 1.4-28³⁹. For mortality and hatching rate, statistically significant differences were evaluated by generalized linear model at the beaker level ($n = 3$), followed by Dunnett's test. Time-weighted average (TWA) concentration was calculated following a previous study¹⁴ to account for concentration changes during exposure.

RESULTS

Exposure concentration

Measured concentrations of 7PPD-Q and 77PD-Q were generally close to their nominal values and remained relatively stable throughout the exposure period, with TWA concentrations ranging from 83% to 118% for 7PPD-Q and 90% to 121% for 77PD-Q (Table S1). However, a greater reduction in measured concentration (up to 35%) was observed for 7PPD-Q at the highest nominal level (2000 µg/L), possibly due to the precipitation of 7PPD-Q (Figures 2E and 2F). Given the predicted aqueous solubility of these chemicals (Table 1), this observed precipitation is reasonable. The measured water quality parameters were within acceptable ranges²⁴: temperature 25.2–26.4°C, pH 8.2–8.6, dissolved oxygen 8.5–9.3 mg/L, and conductivity 318–376 mS/m.



Survival and developmental effects

Survival rates at 8 days post fertilization in the negative and DMSO controls were 100%. Either 7PPD-Q or 77PD-Q did not cause significant reduction in 8-day survival rate even at the highest concentrations, with the average survival rate > 85% (Figure 1).

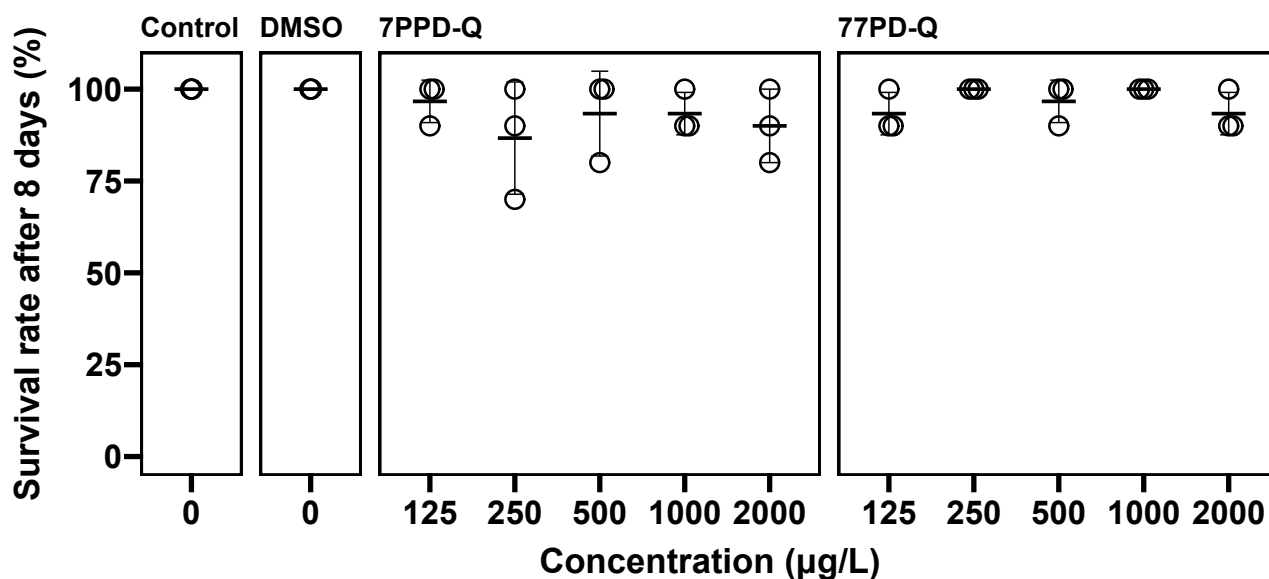


Figure 1. Effects of 7PPD-Q and 77PD-Q on 8-day survival rate of zebrafish embryos. Bars and error bars represent mean values and standard deviations ($n = 3$), respectively.

Hatching rate was statistically significantly reduced only at 96 hpf in the 2000 µg/L 7PPD-Q treatment ($p = 0.0014$), while no significant changes were observed in the other treatments, and average hatching rates reached $\geq 90\%$ in all the treatments (Figure 2A). Although a statistically significant decrease in heartbeat rate at 54–56 hpf was observed at 250 µg/L ($p = 0.047$) and 500 µg/L of 7PPD-Q ($p < 0.001$), the effect was not concentration-dependent and was not observed for 77PD-Q (Figure 2B).

A concentration-dependent reduction in eye size was observed for 7PPD-Q, with statistically significant effects at 1000 and 2000 µg/L ($p < 0.01$) (Figure 2C). Similarly, head size was significantly reduced by 7PPD-Q at ≥ 500 µg/L ($p < 0.01$) (Figure 2D). In contrast, 77PD-Q did not



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4 induce significant changes in either eye or head size. The ratio of eye to head size was not
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6 significantly affected by exposure to either 7PPD-Q or 77PD-Q (Figure S1).
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9 Morphological abnormalities were observed in embryos for exposed to 7PPD-Q and 77PD-Q,
10 including pericardial edema (Figures 2H and 2J), yolk edema (Figure 2H), lordosis (Figure 2H),
11 blood congestion (Figure 2I). However, these abnormalities were limited to a small number of
12 individuals (Table S2), with frequencies averaging < 20% in each treatment. In addition, other
13 morphological abnormalities in zebrafish embryos, previously reported in response to 6PPD
14 exposure, such as uninflated swim bladder and deformed otolith^{28,29}, were not clearly observed in
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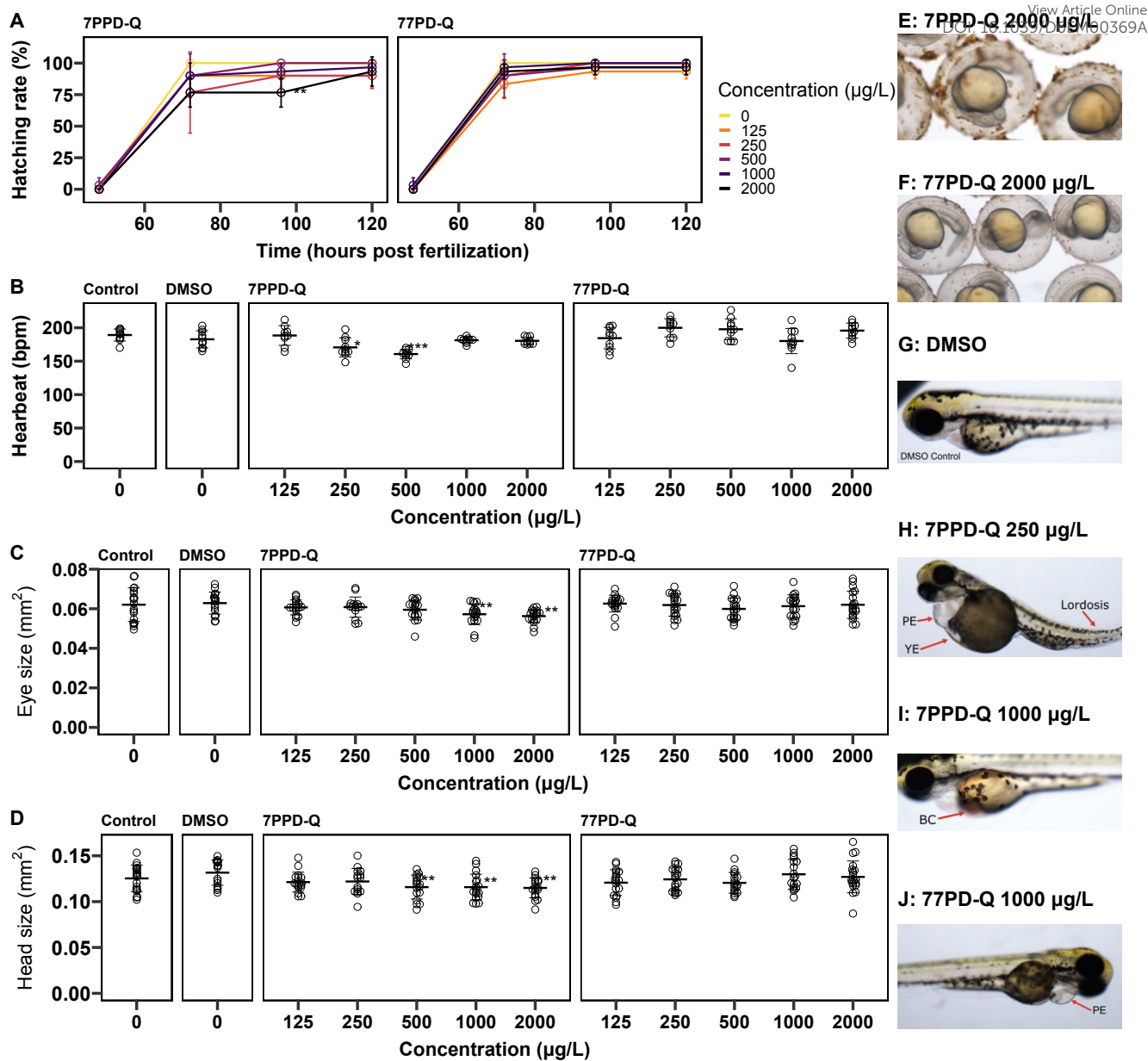


Figure 2. Effects of 7PPD-Q and 77PD-Q on the development of zebrafish embryos. (A) Hatching rate of embryos from 48 to 120 hpf. (B) Heartbeat rate at 54–56 hpf. (C) Eye size at 72 hpf. (D) Head size at 72 hpf. Bars and error bars represent mean values and standard deviations ($n = 3$), respectively. Asterisks represent statistically significant differences from the DMSO control (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$). (E–J) Representative images of embryos. (E) Embryos at 24 hpf at 2000 µg/L of 7PPD-Q. (F) Embryos at 24 hpf at 2000 µg/L of 77PD-Q. (G) Embryo at 72 hpf in the DMSO control. (H) Embryo at 72 hpf at 250 µg/L of 7PPD-Q. (I) Embryo at 72 hpf at 1000 µg/L of 7PPD-Q. (J) Embryo at 72 hpf at 1000 µg/L of 77PD-Q. PE indicates



pericardiac edema, YE indicates yolk edema, and BC indicates blood congestion. View Article Online
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Pigmentation rate in yolk-sac region showed a concentration-dependent reduction for both 7PPD-Q and 77PD-Q, with statistically significant effects at $\geq 1000 \mu\text{g/L}$ for both 7PPD-Q and 77PD-Q ($p < 0.01$) (Figure 3). However, the reduction in pigmentation rate was less than 27% compared to the DMSO control and was not visually apparent (Figure 3).

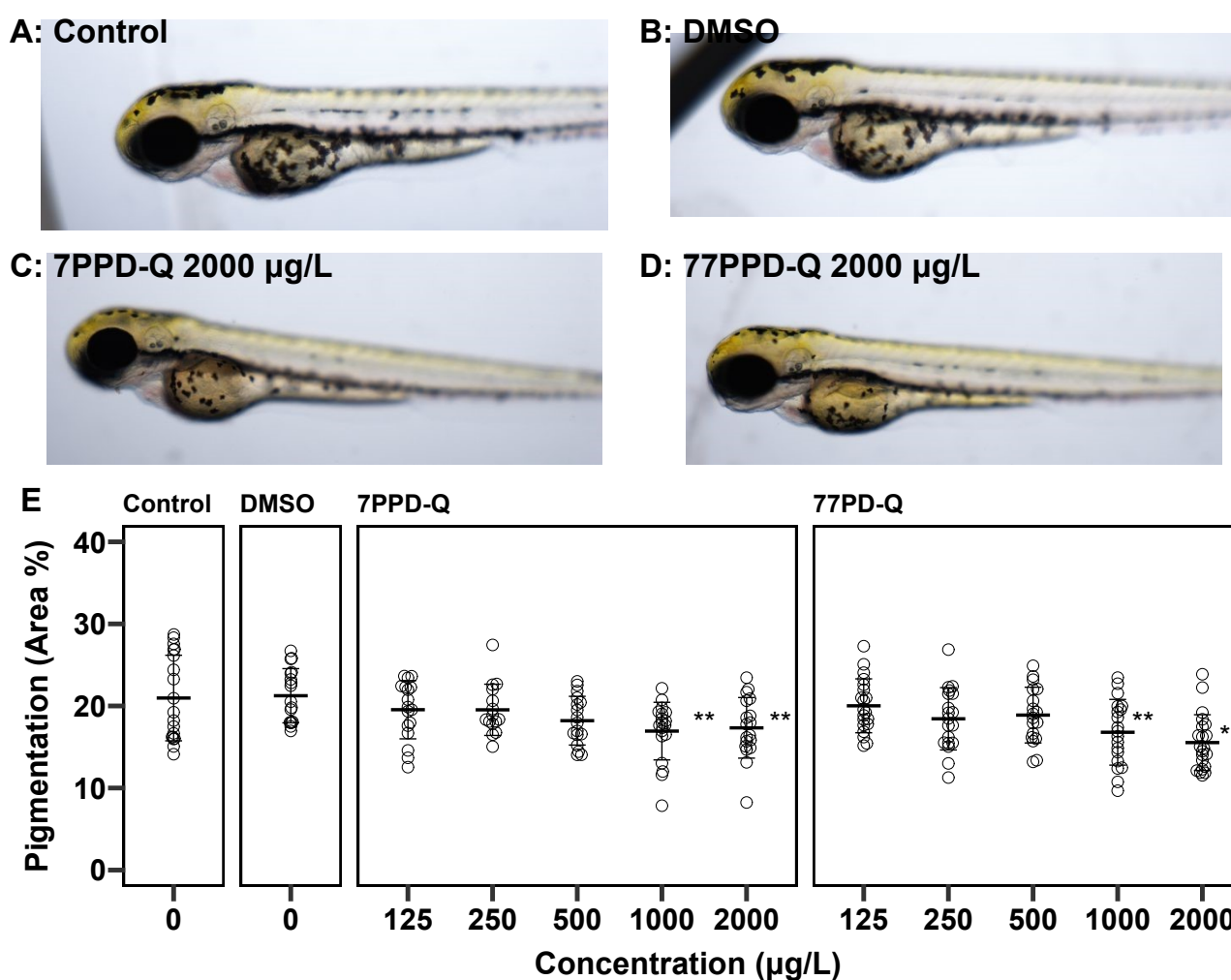


Figure 3. Effects of 7PPD-Q and 77PD-Q on embryo pigmentation rate in yolk-sac region at 72 hpf.

(A–D) Representative images of embryos. Embryos (A) in the control, (B) in the DMSO control, (C) at 2000 $\mu\text{g/L}$ of 7PPD-Q, and (D) at 2000 $\mu\text{g/L}$ of 77PD-Q. (E) Asterisks represent statistically significant differences from the DMSO control (*: $p < 0.05$, **: $p < 0.01$, ***: $p <$

0.001).

After 8 days of exposure, significant reduction in body length was not observed for either 7PPD-Q or 77PD-Q at any tested concentrations (Figure 4). The reduction in body length was less than about 1.8% relative to the DMSO control (3.82 ± 0.17 mm). In addition, across all treatments, all surviving individuals exhibited inflated swim bladders after 8 days of exposure.

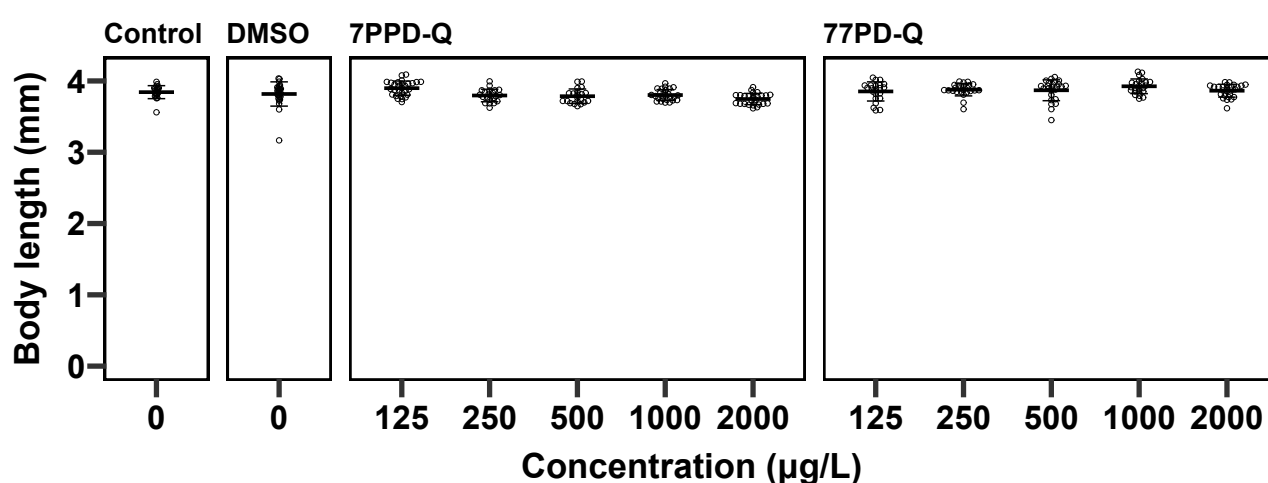


Figure 4. Effects of 7PPD-Q and 77PD-Q on body length of sac-fry at 8 dpf.

DISCUSSION

As quinone transformation products of alternative candidates to tire rubber antioxidant 6PPD, the lethal and sub-lethal toxicity of 7PPD-Q and 77PD-Q were evaluated using zebrafish embryos and sac-fry. We found that neither chemical induced 8-day mortality in zebrafish up to a nominal concentration of 2000 µg/L. However, 7PPD-Q induced significant effects on hatching rate, eye size, head size, and yolk-sac pigmentation, while 77PD-Q induced significant effects on only pigmentation. Other effects, such as heartbeat rate, body length, and morphological changes, were not significant or not concentration-dependent. The lack of acute lethality at or above water solubility limits suggests that the pronounced interspecific sensitivity documented for 6PPD-Q^{11,12}



may also hold for 7PPD-Q, given the 24-h LC₅₀ reported for coho salmon juveniles (5.4 µg/L).

The observed effects of 7PPD-Q were generally consistent with those reported for 6PPD-Q in previous zebrafish studies. According to a study²⁵, 6PPD-Q induced low pigmentation but did not cause other phenotypic changes, such as abnormal eye formation, uninflated swim bladder, irregular heartbeat, or edema, although some effects on eye size and heartbeat have been reported by other studies²⁸. Chang et al. also reported that 6PPD-Q at concentrations up to 2000 µg/L did not affect hatching rate, eye size, body length, and swim bladder morphology²⁹, which is consistent with our observations for 7PPD-Q. Low pigmentation in zebrafish embryos has been associated with disruption of the thyroid system^{25,40}. However, thyroid disruption is also known to cause reduced eye size²⁵ and inhibit swim bladder inflation⁴¹.

In addition, although the effects observed following exposure to 7PPD-Q were broader than those of 77PD-Q, they were also qualitatively similar to findings reported for 6PPD-Q^{25,28} and not consistent with a typical thyroid-disruption profile, given the absence of impaired swim-bladder inflation. Therefore, the observed effects of both 7PPD-Q and 77PD-Q are likely attributable to mechanisms other than thyroid-disruption.

While 7PPD-Q and 77PD-Q concentrations in surface water have rarely been reported⁴², available evidence suggests they are far below the effect concentrations for zebrafish. This is because 6PPD-Q in creeks and surface waters typically occurs at ng/L levels^{2,42,43}, and 6PPD and 6PPD-Q concentrations in water, air, and soil are higher than or comparable to those of other PPDs and PPD-quinones^{7,36,44}. In addition, 7PPD-Q was not detected in surface waters, whereas 6PPD-Q concentrations in the same samples exceeded LC50 for coho salmon (i.e., > 41 ng/L)⁴². Although future environmental concentrations of 7PPD-Q and 77PD-Q after replacement cannot be predicted with confidence, the currently available evidence indicates that these chemicals are unlikely to pose severe developmental risks to zebrafish under typical environmental conditions. Given that short-term toxicity tests with embryo and sac-fry stages are generally less sensitive than full life-cycle chronic tests (OECD, 1998), further studies are recommended to investigate the chronic effects of

these chemicals.

CONCLUSIONS

This study evaluated the lethal and sublethal toxicity of the quinone transformation products of proposed 6PPD alternatives—7PPD-Q and 77PD-Q—using zebrafish embryos and sac-fry under OECD-aligned conditions. Exposure was verified and largely stable across treatments, with visible precipitation only at the highest nominal concentration. Neither chemical caused 8-day mortality up to 2000 µg/L. However, 7PPD-Q produced clear, concentration-related developmental effects: reduced eye and head size (detectable ≥ 500 µg/L), modest hatching suppression at 2000 µg/L, and slight low pigmentation in the yolk-sac region. By contrast, while 77PD-Q primarily decreased pigmentation at ≥ 1000 µg/L, effects on hatching, eye and head size, body length, heartbeat, and gross morphology were not significant or not concentration-dependent. Across all treatments, surviving individuals exhibited inflated swim bladders, and the eye-to-head size ratio was unchanged, suggesting that the observed responses do not follow a canonical thyroid-disruption profile. Considering that the effect ranges observed in this study are far above currently reported environmental levels of these quinones and are close to or above their predicted water solubility limits, acute developmental risk to zebrafish under typical conditions appears low.

Conflict of interest

There are no conflicts of interest to declare.

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Author contribution statement

Kyoshiro Hiki: Conceptualization; Methodology; Investigation; Formal Analysis; Visualization; Funding acquisition; Writing—original draft; Writing—review & editing. **Chenyang Rao:** Conceptualization; Methodology; Investigation; Formal Analysis; Writing—review & editing. **Riping Huang:** Methodology; Investigation; Writing—review & editing. **Hiroshi Yamamoto:** Supervision; Resources; Writing—review & editing.

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Data availability statement

Zebrafish embryo images are available at GitHub repository (https://github.com/KyoHiki/zebrafish_7PPDQ_77PDQ/). The other data supporting this paper have been included as part of the Supplementary Information.

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