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Embryotoxic Effects of Para Substituted Chloro-Nitro Phenol Compounds on Zebrafish

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Abstract

Background: Found on the priority organic pollutants list and mono substituted derivatives of phenol, 4-chloro and 4-nitrophenol compounds are organic-origin micropollutants with toxicologic research performed due to recalcitrant properties, endocrine disrupting and lipophilic character, and tendency for bioaccumulation. As both pollutants are used in many industrial and other applications (from paper, pesticides, dye, pharmaceutical and explosives industries to chlorination of drinking water), they rapidly accumulate at all trophic levels of the ecosystem and are known to cause negative effects on organisms in the ecosystem led by aquatic systems. As a result, there are many methods developed for toxicity research of these types of priority organic pollutants, though studying the small freshwater fish species of zebrafish (embryo and adult) is chosen due to some advantages compared to other methods.

Aims: To our knowledge, there are no studies performed on the embryotoxic effects of 4-nitrophenol and 4-chlorophenol derivatives on fish species. With this aim, the lethal and teratogenic effects of the phenol derivatives on zebrafish (*Danio rerio*) were evaluated by means of FET (fish embryo toxicity) test.

Materials and methods: According to FET test, embryos at 1.5 hpf (hour post-fertilization) were exposed to 4-nitrophenol and 4-chlorophenol solutions in concentrations 1.0, 2.0, 4.0, 8.0 and 16.0 mg/L during 96 h. Every 12 hours, the embryos were observed and scored for lethal and teratogenic effects. Also, LC50 values were calculated for 48 h.

Results: 48 h LC50 values were 2.409 mg/L for 4-chlorophenol and 4.581 mg/L for 4-nitrophenol. With increasing phenol concentrations, lethality and malformations increased. Teratogenic malformations more frequently produced by phenol derivatives were: incomplete body, chorda deformity, tail curvature, lordosis, scoliosis, delayed development and hatching, weak or non-pigmentation.

Conclusion: 4-chlorophenol was found to be more toxic than 4-nitrophenol. Results firstly showed that the phenol derivatives caused embryotoxicity in zebrafish.

Keywords: 4-chlorophenol; 4-nitrophenol; Zebrafish; Embryotoxicity, LC50

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Introduction

Currently, in parallel with developments in industry and technology, the amount of waste polluting the environment in the form of solid-liquid-gas has rapidly increased, whether due to the numbers of chemicals entering production or linked to the production capacity. These pollutants (organic/inorganic) have toxic and ecotoxic

effects linked to their origin. In addition to direct toxic effects on humans and animals, the ecotoxic effects caused linked to distribution and variation processes within the ecosystem (water-soil-air-sediment) have reached dimensions that cannot be ignored.[1] Additionally, it is a known reality that the split in the correlation between ecological footprint and industrial production has opened to a degree that cannot be compensated for and this is an important criterion showing the degree to which toxic chemicals have polluted the ecosystem.

Intensely used in agriculture, industry and even disease control, the important chemicals of chlorinated and nitrated phenol compounds are known to cause both health and environmental problems at serious dimensions.[2] Especially 4-chloro/nitro phenol compounds, with high logKow (bioaccumulation marker) value and resistant to biodegradation, accumulate in aquatic environments and negatively affect aqueous organisms.[3]

Chlorinated phenol compounds are organic phenol derivatives with many alternative derivatives containing from one (mono) to five (penta) chlorine atoms. Chlorophenols (CP) are listed among the priority toxic pollutants according to the US EPA, and are organic chemicals that have the ability to change bacterial activity, phytotoxicity and bioaccumulation capability linked to the number and position of chlorine atoms and which can be found in surface waters, groundwater and even drinking water (disinfected with chlorine).

As a result, the mixing and accumulation percentage of chlorophenols in the environment is very high.[4] In addition to use in wood, dye, tanning and disinfectant, they are commonly encountered in many industrial processes and byproduct synthesis.

These include the use of chlorophenols as raw materials in production of herbicides, fungicides, insecticide, pharmaceuticals and dyes, and their use as organic solvents. They may also be released from burning of waste with high organic content, chlorine paper-bleaching processes and dechlorination of drinking waters.[5]

Among chlorophenols, the most toxic mono-chlorophenol derivative of (para) 4-chlorophenol (4-CP) is a congener of this group and in addition to being included on the priority pollutant list, it is an organic pollutant known to be teratogenic, carcinogenic, and mutagenic for organisms and very toxic.[6]

Nitrophenols, similar to chlorophenols, are other substituted phenols with alternative derivatives from mono to penta. Nitrophenol (NP) compounds are commonly used in production of pesticides, dyes and dye material, plastic, rubber, explosive materials, tanning and pharmaceuticals. NPs present in industrial wastewater have high refractivity, high stability and water-soluble characteristics, in addition to being released from diesel exhausts.[7]

Like other substituted phenol derivatives, nitrophenols have abundant sources in the environment and are pollutants with high probability of being found in surface water, groundwater (rain water, snow melt, rivers and sediments) and waste water.[8] There are 3 different nitrophenol derivatives (2-NP, 4-NP and 2,4-DNP) present on the Environmental Protection Agency (EPA) priority pollutant list.[9]

Among these NP compounds, the very toxic mono nitrophenol of 4-NP is resistant to biological degradation, and rapidly accumulates at trophic levels in aquatic and terrestrial organisms. 4-NP was identified in the urine of people exposed to organophosphate pesticides, while it displays EDC (Endocrine Disruptive Compound) effects in rodents.[10] There are many studies showing this compound, resistant to biodegradation, is toxic.[11]

Some substituted phenol derivatives are known to have toxic effects on invertebrate and vertebrate animal species. Many studies have shown that these compounds have known genotoxic effects in both humans and animals, while the studies related to embryotoxicity are very limited.[12,13]

Zebrafish (*Danio rerio*) are an important model organism used in toxicity research. The embryos are very commonly used to determine the developmental toxicity of many chemicals.[14–17] The embryos are transparent, and every embryonic stage is easily visible.

Also, they have high fecundity, and can be produced by external fertilization so they are appropriate for *in vitro* studies. They have homologous gene structure to humans (xenobiotic metabolism phase I and phase II enzyme systems) making zebrafish a model organism with perfect compliance for FET (Fish Embryo Toxicity) tests. The aim of the present study was to determine the probable embryotoxic effects of phenol derivatives with substitution in the para position of 4-CP and 4-NP by using zebrafish embryos with the FET test.

Materials and Methods

Chemicals

4-nitrophenol (4-NP) and 4-chlorophenol (4-CP) compounds were obtained from Sigma. Stock solutions were separately prepared for each compound. 4-CP was dissolved in water, while 4-NP was dissolved in 1% DMSO. Stock solutions were stored at 5°C until the start of experiments.

Fish culture and eggs production

Adult zebrafish were obtained from Atatürk University, Faculty of Aquaculture, Aquarium Fish Research Center. Before experiments, fish were left to adjust to the new environment for 14 days. Twenty healthy adult fish were placed in the aquarium and photoperiod was set to 14:10 light/dark cycles. Dechlorinated water was placed in the aquaria, and the water temperature was set to 27 ± 1°C.

The aquarium water had 1/3 of the water changed 1 time per week. Fish were fed with flake food 2 times per day. One week before obtaining eggs, the separated female and male fish were placed in a breeding aquarium. With fertilization occurring in the early hours of the morning, fertilized eggs were removed from the aquarium and washed twice in distilled water. The fertilized eggs were later placed in petri dishes containing Hank's solution and placed in an incubator set to 27 ± 1°C.

Embyrotoxicity Test

The FET test is a standard test coded OECD TG236 based on monitoring of embryo development at certain intervals after contact of fertilized fish embryos with a probable toxicant.[18] 4-NP and 4-CP phenol derivatives were analyzed according to this test. Firstly, previous studies were used to determine the experimental concentrations.[16,19] The control group used Hank's solution, while the negative control group used a solution containing 1% DMSO.

At approximately 1.5 hours post-fertilization (hpf), embryos in the incubator were placed in petri dishes containing 1.0, 2.0, 4.0, 8.0 and 16.0 mg/L substituted phenol derivatives, and the 96 hours exposure was begun after the fertilized eggs were placed into the test solutions. Each petri dish contained 20 fertilized fish embryos and the experiment had 3 repeats (n = 60).

The solutions in the petri dishes were changed at 24-hour intervals. Every 12 hours, the embryos were observed. Dead embryos were immediately removed and recorded.

The fish embryos in 48-hpf; especially, were used to determine the median lethal dose (LD50) of the compounds. Teratogenic abnormalities like coagulation, edema, lordosis, etc. observed in fish embryos and larva were identified and photographed with a digital camera device. Teratogenic abnormalities were classified according to the grading reported by Padilla et al.[20] Using this grading, the total malformation index (MI) was calculated. The group with MI values from 0 to 3 were normal, the group with values from 4 to 6 were slightly abnormal, and the group with values of 7 and above were accepted as completely abnormal larva.

Statistical Analysis

Statistical analysis was performed using the SPSS 20.0 software programme. The 48-hpf LC50 value was calculated with Probit analysis. Evaluation of abnormal embryos and larvae used the one-way ANOVA test. All data are given as mean ± standard deviation. Multiple comparisons had the Dunnett test performed after variance analysis and statistical significance level was accepted as $p < 0.05$.

Results

The 48-hpf LC50 values were calculated as 2.409 mg/L for 4-chlorophenol and 4.581 mg/L for 4-nitrophenol. As the phenol concentration increased, it was found the number of deaths and abnormal embryos increased. It was observed that 4-CP was more toxic than 4-NP. The calculated LC50 values are reported for the first time in this study (Fig. 1 and 2).

The embryo development slowed down in the phenol groups (Table 1). The developmental parameters such as gastrulation, somite formation, optical vesicle formation, spontaneous movement, tail separation, heartbeat, blood circulation, pigmentation and hatching regressed at sub-lethal phenol doses, while some appeared not to fully occur. Phenols additionally caused a reduction in the number of hatching larvae. The larvae which never hatched with high phenol concentrations at the end of exposure died after a while.

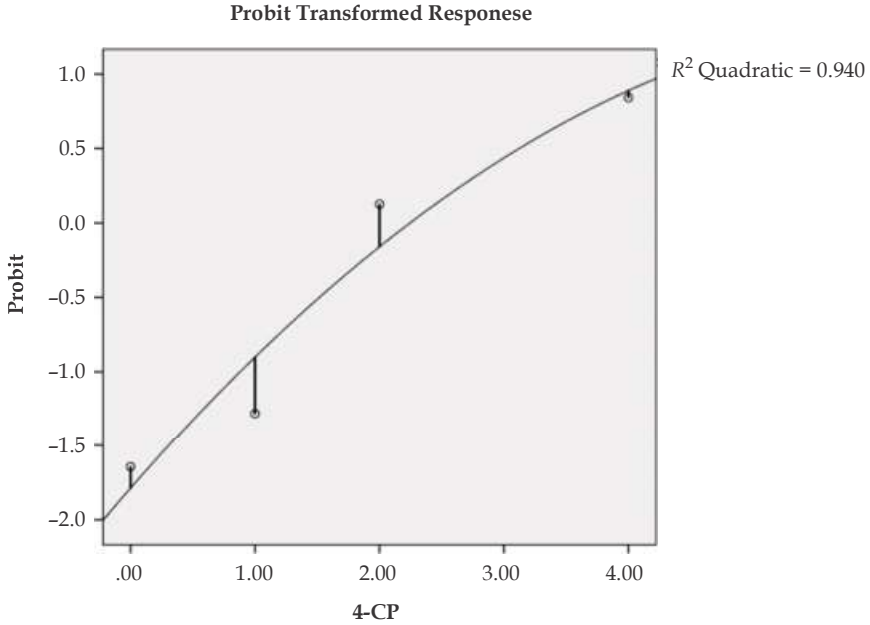

Fig. 1: Dose-response curve used for calculation of LC50 value for 4-CP (confidence interval 95%).

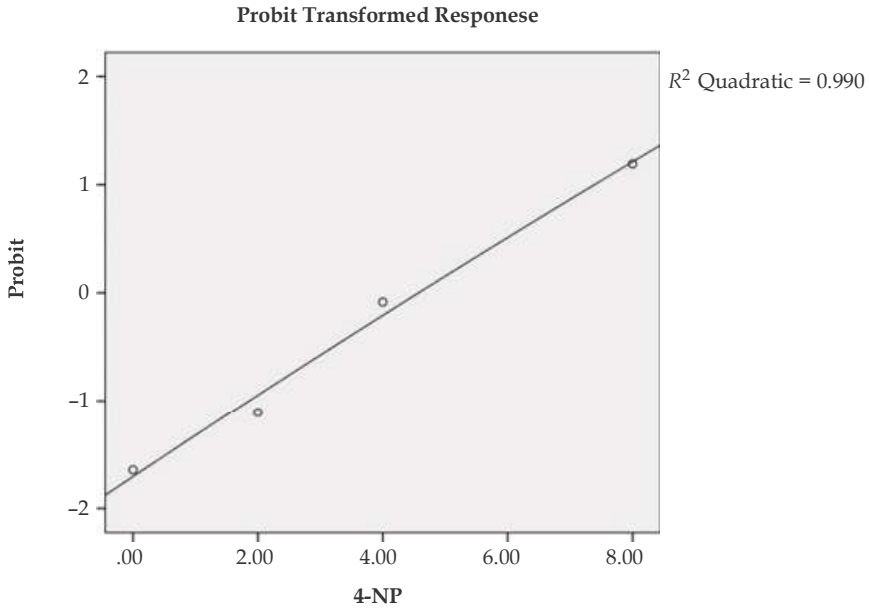

Fig. 2: Dose-response curve used for calculation of LC50 value for 4-NP (confidence interval 95%).

Table 1. Developmental rates of the embryos exposed to the phenols

Developmental period[a] Hour[b]	Cleavage 1.5 hpf	75% Epiboly 8 hpf	Pharyngula 24 hpf	Hatching 48 hpf	Larva 80 hpf
Control	1.6 ± 0.10	8.7 ± 0.20	24.5 ± 1.20	49.3 ± 1.60	82.0 ± 1.30
DMSO (%1)	1.6 ± 0.05	9.0 ± 0.10	26.6 ± 0.80	47.2 ± 1.80	84.1 ± 1.90
4-CP (2 mg/L)	1.7 ± 0.10	17.2 ± 1.10*	39.4 ± 2.80*	76.2 ± 6.30*	120.2 ± 8.60*
4-NP (4 mg/L)	1.7 ± 0.10	16.0 ± 0.90*	36.5 ± 2.20*	69.4 ± 7.40*	111.3 ± 8.80*

[a]Stages given according to Kimmel et al. [21][b]hpf: hour post-fertilization. *shows differences at $p < 0.05$ level compared to control. Values given as mean ± standard error.

At high phenol doses, more than half of fish embryos died within the first 48 hours. The teratogenic effects observed over 96-hours duration in embryos and the mortality rates are given in Tables 2 and 3. As phenol concentrations increased, the number of affected embryos and larvae increased. With exposure to 8 and 16 mg/L 4-CP, all embryos died. At the end of 96 hours in the 2 and 4 mg/L the phenol groups, the majority of surviving embryos (76% and 86% respectively) were observed to have teratogenic abnormalities (Table 2).

Table 2: Adverse effects in embryos caused 4-CP at 96-hour exposure

	Control	1 mg/L	2 mg/L	4 mg/L	8 mg/L	16 mg/L
Number of teratogenic embryos	2	4	13	6	0	0
Number of dead embryos	3	6	33	48	60	60
Number of affected embryos	5	10	46	54	60	60
Number of normal embryos	55	50	14	6	0	0
% Teratogenic embryos	3.3 ± 0.1	6.6 ± 0.5	21.6 ± 3.0	6.6 ± 0.9	0	0
% Dead embryos	5.0 ± 0.2	10 ± 1.0	55 ± 8.2	80 ± 5.6	100 ± 0	100 ± 0
% Affected embryos	8.3 ± 1.0	16.6 ± 2.0*	76.6 ± 9.7*	86.6 ± 6.7*	100 ± 0*	100 ± 0*
% Normal embryos	91.7 ± 5.8	83.4 ± 5.0	23.4 ± 4.6	13.4 ± 1.4	0	0

*Shows differences at $p < 0.05$ level compared to control

All embryos died at 16 mg/L 4-NP dose. At the end of 96 hours, in the 4 and 8 mg/L the phenol groups, most of the surviving embryos (79% and 86%) were observed to have teratogenic abnormalities (Table 3).

Table 3: Adverse effects in the embryos caused 4-NP at 96-hour exposure

	Control	DMSO	1 mg/L	2 mg/L	4 mg/L	8 mg/L	16 mg/L
Number of teratogenic embryos	2	1	3	7	20	2	0
Number of dead embryos	3	3	2	8	28	53	60
Number of affected embryos	5	4	5	15	48	55	60
Number of normal embryos	55	56	55	45	12	5	0
% Teratogenic embryos	3.3 ± 0.1	1.6 ± 0.1	5.0 ± 1.0	11.6 ± 1.5	33.3 ± 6.5	3.3 ± 0.2	0
% Dead embryos	5.0 ± 0.2	5.0 ± 0.3	3.3 ± 0.2	13.3 ± 2.6	46.6 ± 6.6	83.3 ± 5.3	100 ± 0
% Affected embryos	8.3 ± 1.0	6.6 ± 0.6	8.3 ± 0.7	24.9 ± 3.8*	79.9 ± 8.5*	86.6 ± 4.8*	100 ± 0*
% Normal embryos	91.7 ± 5.8	93.4 ± 6.0	91.7 ± 6.6	75.1 ± 8.4	20.1 ± 2.5	13.4 ± 2.1	0

*Shows differences at $p < 0.05$ level compared to control.

Fish embryos exposed to lethal and sub-lethal phenol doses within 48 hours were observed to have different teratogenic effects such as incompleted head, eye, tail and chorda (Fig. 3B), weak pigmentation, chorda defects and lack of body parts (Fig. 3D). In the same way, 72-hpf larvae had chorda abnormalities such as lordosis (Fig. 4B), tail curvature (Fig. 4C) and scoliosis (Fig. 4D) linked to increasing phenol concentrations. Additionally, non-hatching larvae and non-pigmentation embryos were identified (Fig. 5). These abnormalities were not observed (Fig. 3A, Fig. 3C, Fig. 4A) or observed less in fish embryos and larvae in the control and negative control groups. The most dominant abnormalities were detected as chorda malformations. All of teratogenic embryos and larvae died within 1–2 days after 96 hpf.

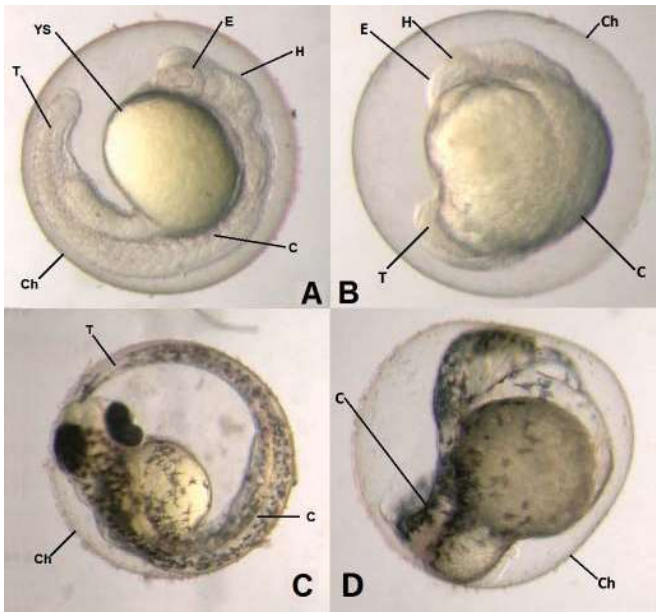

Fig. 3: Normal fish embryos at 24 and 48 hpf and abnormal embryos exposed to 4 mg/L 4-CP and 8 mg/L 4-NP. (A) 24-hpf normal embryo, (B) 24-hpf abnormal embryo; incompleted head, eye, tail and chorda, (C) 48-hpf normal embryo, (D) 48-hpf abnormal embryo; weak pigmentation, chorda defects and lack of body parts. Ch: chorion, C: chorda, E: eye, H: head, T: Tail, YS: yolk sac.

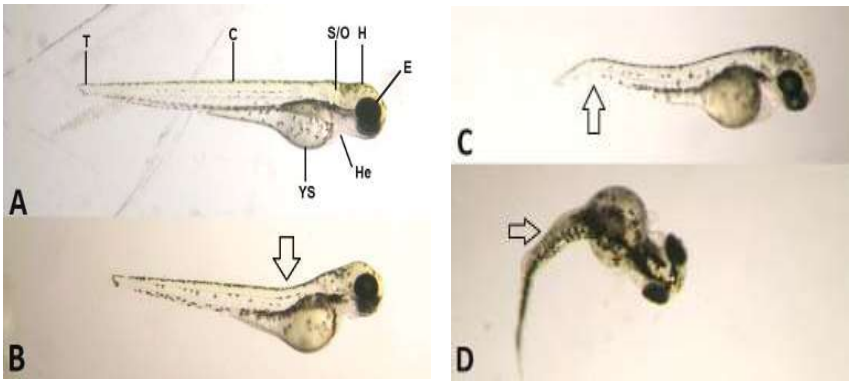

Fig. 4: 72-hpf normal fish larva and abnormal larvae exposed to 4 mg/L 4-CP and 8 mg/L 4-NP. (A) 72-hpf normal larva, (B) 72-hpf abnormal larva; lordosis (arrow), (C) 72-hpf abnormal larva; tail curvature (arrow), (D) 72-hpf abnormal larva: scoliosis (arrow). C: chorda, E: eye, H: head, He: heart, S/O: sacculus/otolith, T: Tail, YS: yolk sac.

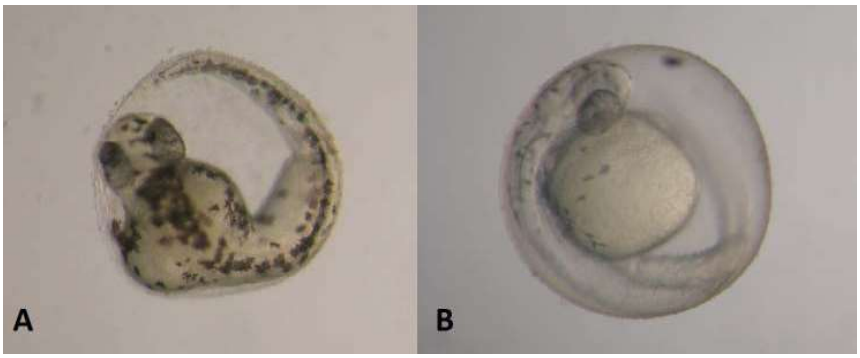

Fig. 5. Abnormal fish embryos exposed to 4 mg/L 4-CP and 8 mg/L 4-NP. (A) 72-hpf non-hatching larva, (B) non-pigmentation 48-hpf embryo.

The total malformation index (MI) values for the phenol compounds are presented in Table 4. The MI value for 1 mg/L phenol dose was not different to the control and DMSO groups. However, the MI values for 2 mg/L, 4 mg/L and 8 mg/L phenol concentrations increased. Especially for 4 mg/L 4-CP and 8 mg/L 4-NP doses, the MI values were above 7 indicating the most embryos and larvae were deformed. The MI values for 8 and 16 mg/L 4-CP and for 16 mg/L 4-NP could not be calculated.

Table 4. Total malformation index (MI) values calculated for 72-hour fish larvae exposed to the phenol derivatives

	MI values				
Control			1.7 ± 0.2		
DMSO			1.9 ± 0.2		
	1 mg/L	2 mg/L	4 mg/L	8 mg/L	16 mg/L
4-CP	2.7 ± 0.7	5.6 ± 0.6*	8.0 ± 1.0*	0	0
4-NP	2.3 ±0.6	4.5 ± 0.4*	6.2 ± 0.8*	7.6 ± 1.6*	0

* Show differences at $p < 0.05$ level compared to control

Discussion

Death and various teratogenic abnormalities were observed linked to increasing concentration with exposure of zebrafish embryos to 4-CP and 4-NP for 96 hours. For both compounds, the 48-hour LC50 values were calculated as 2.409 mg/L for 4-CP and 4.581 mg/L for 4-NP. These results comply with the results of other studies. The 96-hour LC50 value for 4-CP for Javanese ricefish (*Oryzias javanicus*) was found to be 4.1 mg/L.[22] Pentochloro-phenol (PCP) had 24- and 48-hour LC50 values of 196 and 130 μg/L for zebrafish,[23] with the 48-hour LC50 values for 2-CP and 2, 4-DCP identified as 8.378 mg/L and 6.558 mg/L for zebrafish embryos.[17] Zhang et al.[24] reported that the 120-hour LC50values for 2,4-DCP and 2,4,6-TCP were 2.45 mg/L and 1.11 mg/L for zebrafish embryos. For nitrophenols the situation is not different. Ceylan et al.[16] reported the 48-hour LC50 values for 2-NP and 2,4-DNP were 18.7 mg/L and 9.65 mg/L for zebrafish embryos. The 48- and 96-hour LC50 values identified for 4-NP for fresh and saltwater organisms varied from 11.0 mg/L to 62 mg/L. For rainbow trout (*Oncorhynchus mykiss*) this value was found to be 78.9 mg/L. For shellfish, this value was identified as 2.8 to 20 mg/L.[25] Nitrophenols have less deadly effect compared to chlorophenols and the USEPA[26] recommends they be present at maximum 3.5 mg/L levels in environmental waters and this is supported by our results.

The substituted phenol concentrations identified in aquatic systems are not yet at toxic levels. The highest identified chlorophenol concentration was 0.002–2 mg/L in river waters. In drinking water, this rate is lower.[27] The amounts of nitrophenols identified in fresh water do not pass 0.0057 mg/L.[28] However, comprehensive monitoring studies continue around the world.

In our study, sublethal doses of 4-CP and 4-NP caused developmental toxicity and a variety of teratogenic abnormalities in zebrafish embryos larvae. Similar results were obtained from previous studies. For example, Xu et al.[29] reported a reduction in neutrophil counts and endocrine system disruptions in 7-day zebrafish larvae with 5 mg/L 4-NP exposure. Osin et al.[19] exposed zebrafish to different doses of 4-NP (1.0, 5.0, 10.0, 15.0 and 20.0 mg/L) and reported that there was a dose-linked increase in embryo deaths and a reduction in the number of hatching larva. There are fewer studies showing the embryotoxic effects of chlorophenols. Xu et al.[30] stated there was developmental regression in 8-hpf zebrafish embryos with PCP exposure. Lopez-Romera et al.[31] observed that Na-PCP caused death of zebrafish embryos within 72 hours and revealed a variety of teratogenic effects.

There is limited information about the toxic effect mechanism of substituent phenols. Phenols are known to reduce the activity of some biochemical enzymes. For example, 2-CP triggers formation of reactive oxygen radicals in fish,[32] while 2,4-DCP causes oxidative stress.[33] Thus, the damage occurs in some important cell molecules such as DNA, protein and lipid.[34] Chlorophenols disrupt the mitochondrial membrane potential[35] and cause cell death leading to acute toxicity.[36] For nitrophenols, after exposure ATP levels in the organism reduce, and it is known that oxidative metabolism is disrupted especially in zebrafish.[37] The lethality and teratogenicity occurring in zebrafish embryos linked to 4-CP and 4-NP may be explained by

increased oxidative stress during embryonic cell development in fish and disruption of oxidative metabolism.

Conclusion

This study showed the adverse effects of 4-CP and 4-NP on the embryonic development of zebrafish. 4-CP was found to be more toxic than 4-NP (nearly 2 times). Substituent phenol doses of 2.0, 4.0 and 8.0 mg/L are estimated to be potentially harmful for aquatic organisms. The level of 4-NP identified in aquatic systems is not yet at levels to affect aquatic organisms, while it has been revealed that 4-CP has reached the margins of critical levels in research. However, if precautions are not taken, it should not be forgotten that these two substituent phenol compounds will increase in natural environments and will affect organisms in the ecosystems.

References

1. Mill T, Mabey W. Photodegradation in water. In: Environmental Exposure from Chemicals. Vol. 1. Neely, W. B., Blau, G. E., Eds. 1985. pp.175–216, CRC Press, Boca Raton, FL.
2. Igbinosa EO, Odjadjare EE, Chigor VN, et al. Toxicological profile of chlorophenols and their derivatives in the environment: the public health perspective. Sci. World J., 2013:460215.
3. Czaplicka M. Sources and transformations of chlorophenols in the natural environment. Science of the Total environment 2004;322(1-3):21–39.
4. Olaniran AO, Igbinosa EO. Chlorophenols and other related derivatives of environmental concern: properties, distribution and microbial degradation processes. Chemosphere. 2011 May;83(10):1297–306.
5. Pera-Titus M, Garcia-Molina V, Banos MA et al. Degradation of cholorophenols by means of advanced oxidation processes: A general review. Applied Catalysis B: Enviromental 2004;47:219–56.
6. Munoz M, Kaspereit M, Etzold BJ. Deducing kinetic constants for the hydrodechlorination of 4-chlorophenol using high adsorption capacity catalysts. Chemical Engineering Journal 2016;285:228–35.
7. Donlon BA, Razo-Flores E, Lettinga G. et al. Continuous detoxification, transformation and degradation of nitrophenols in up flow anaerobic sludge blanket (UASB) reactors. Biotechnology and Bioengineering 1996;51(4):439–49.
8. Pocurull E, Calull M, Farce RM. et al. Determination of phenolic compounds at low _g 1_1 levels by various solid-phase extractions followed by liquid chromatography and diode-array detection. Journal of Chromatography A 1996;719(1):105–12.
9. USEPA. 440177001 Sampling and Analysis Procedures for Screening of Industrial Effluents for Priority Pollutants 1977.
10. Ghosh A, Khurana M, Chauhan A. et al. Degradation of 4-Nitrophenol, 2-Chloro-4-nitrophenol, and 2, 4-Dinitrophenol by *Rhodococcus imtechensis* Strain RKJ300. Environ. Sci. Technol 2010;44(3):1069–77.
11. Chen J, Song M, Li Y. et al. The effect of phytosterol protects rats against 4-nitrophenol-induced liver damage. Environmental Toxicology and pharmacology 2016;4:266–71.
12. Prati M, Biganzoli E, Boracchi M. et al. Ecotoxicological soil evaluation by FETAX. Chemosphere 2000 Nov;41(10):1621–28.
13. Qiao M, Wang C, Huang S. Composition, sources, and potential toxicological significance of PAHs in the surface sediments of the Meiliang Bay, Taihu Lake, China. Environ. Int 2006 Jan;32(1):28–33.
14. Nagel R. DarT: The embryo test with the zebrafish *Danio rerio*: A general model in ecotoxicology and toxicology. Altex 2002;19:38–48.
15. Westerfield M. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (*Danio rerio*). 5th Edition, University of Oregon Press, Eugene 2007.
16. Ceylan Z, Şişman T, Yazıcı Z. et al. Embryotoxicity of nitrophenols to the early life stages of zebrafish (Danio rerio). Toxicology and Industrial Health. 2016;32(8):1414–22.
17. Ceylan Z, Sisman T, Dane H. The Embryotoxicity of Some Phenol Derivatives on Zebrafish, *Danio rerio*. Caspian Journal of Environmental Sciences 2019;17(1):11–22.
18. Braunbeck T, Kais B, Lammer E. et al. The fish embryo test (FET): origin, applications, and future. Environmental Science and Pollution Research. 2015;22(21):16247–61.
19. Osin O, Yu T, Cai X. et al. Photocatalytic degradation of 4-nitrophenol by C, N-TiO2: Degradation efficiency vs. embryonic toxicity of the resulting compounds. Frontiers in Chemistry 2018;6:192.
20. Padilla S, Hunter DL, Padnos B. et al. Assessing locomotor activity in larval zebrafish: Influence of extrinsic and intrinsic variables. Neurotoxicol. Teratol 2011;33:624–30.
21. Kimmel CB, Ballard WW, Kimmel SR. et al. Stages of embryonic development of the

zebrafish. Dev. Dyn 1995;203:253–310.

22. Koyama J, Kawamata M, Imai S. et al. Javamedaka: a proposed new marine test fish for ecotoxicology. Environ. Toxicol 2008:23;487–91.
23. Yin D, Gu Y, Li Y. et al. Pentachlorophenol treatment *in vivo* elevates point mutation rate in zebrafish *p53* gene. Mut. Res 2006;609:92–101.
24. Zhang Y, Liu M, Liu J. et al. Combined toxicity of triclosan, 2, 4-dichlorophenol and 2, 4, 6-trichlorophenol to zebrafish (*Danio rerio*). Environ. Toxicol. Pharmacol 2018;57:9–18.
25. ANZECC & ARMCANZ Australian and New Zealand Guidelines for Fresh and Marine Water Quality, Australian and New Zealand Environment and Conservation Council and Agriculture and Resource Management Council of Australia and New Zealand, Canberra. 2000. WEB: https://www.waterquality.gov.au/anz-guidelines/resources/previous-guidelines/anzecc-armcanz-2000.
26. USEPA. Generalized methodology for Conducting Industrial reduction evaluations TREs, EPA 600/2-88/070.1989.
27. Michaleowicz J. The occurrence of chlorophenols, chlorocatechols and chlorinated methoxyphenols in drinking water of the largest cities in Poland. Polish Journal of Environmental Studies 2005;14(3):327–33.
28. Heudorf U, Angerer J, Drexler H. Current internal exposure to pesticides in children and adolescents in Germany: Blood plasma levels of pentachlorophenol (PCP), lindane (γ-HCH), and dichloro (diphenyl) ethylene (DDE), a biostable metabolite of dichloro (diphenyl) trichloroethane (DDT). International journal of hygiene and environmental health 2003;206(6):485–91.
29. Xu H, Zhang X, Li H. et al. Immune response induced by major environmental pollutants through altering neutrophils in zebrafish larvae. Aquatic Toxicology 2018;201:99–108.
30. Xu T, Zhao J, Hu P. et al. Pentachlorophenol exposure causes Warburg-like effects in zebrafish embryos at gastrulation stage. Toxicol Appl Pharmacol. 2014 Jun 1;277(2):183–191.
31. López-Romero F, Zú˜niga G, Martínez-Jerónimo F. Asymmetric patterns inthe cranial skeleton of zebrafish (*Danio rerio*) exposed to sodium pentachlorophenate at different embryonic developmental stages. Ecotoxicol. Environm. Saf 2012;84:25–31.
32. Luo Y, Su Y, Wang XR. et al. 2-chlorophenol induced ROS generation in freshwater fish *Carassius auratus* based on the EPR method. Chemosphere. 2006 Nov;65(6):1064–73.
33. Han SK, Ichikawa K, Utsumi H. Quantitative analysis for the enhancement of hydroxyl radical generation by phenols during ozonation of water. Water Res 1998;32:3261–66.
34. Murray AR, Kisin E, Castranova V. et al. Phenol-induced *in vivo* oxidative stress in skin: Evidence for enhanced free radical generation, thiol oxidation, and antioxidant depletion. Chem Res Toxicol. 2007 Dec;20(12):1769–1777.
35. Danzo BJ, Shappell HW, Banerjee A. et al. Effects of nonylphenol, 1, 1-dichloro-2, 2-bis (p-chlorophenyl)ethylene (p,p′-DDE), and pentachlorophenol on the adult female guinea pig reproductive tract. Reprod Toxicol. 2002 Jan-Feb;16(1):29–43.
36. Matsumura H, Matsuoka M, Igisu H. et al. Cooperative inhibition of acetylcholinesterase activities by hexachlorophene in human erythrocytes. Arch. Toxicol 1997;71(3):151–56.
37. Marit JS, Weber LP. Acute exposure to 2,4-dinitrophenol alters zebrafish swimming performance and whole body triglyceride levels. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 2011;154(1):14–18.