Chronic Toxic Effect of Phenol on Zebrafish *Branchydanio rerio*Hossein Razani,*¹ Kenji Nanba,*² and Shiro Murachi*²

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Duplicated groups of 9 zebrafish, 3 females and 6 males, were exposed to 2.2, 4.9 and 24 ppm phenol solutions for 3 months. The fish behaved abnormally at 4.9 and 24 ppm almost immediately after placement in the test aquaria. The effect of phenol toxicity on the gill, ovary, kidney, intestine, and blood cells were studied carefully. The extent of damage of these organs and also the abnormal behavior were more severe at 24 ppm than at 4.9 ppm. Histological pictures of the studied organs and behavior of the fish at 2.2 ppm were almost similar to those at controls.

Most of the fish at 24 ppm died within a few days, but some remained alive till the end of the experiment. However, no fish died at 4.9 and 2.2 ppm. The survived fish gradually recovered from the initial damage and showed improved condition till the end of the experiment.

None of the fish spawned at 24 ppm, though all the fish at 2.2 and 4.9 ppm spawned frequently. The interval between spawnings were longer at 2.2 and 4.9 ppm than at control groups.

The off-spring (eggs and larvae) of 4.9 ppm which were cultured in the same concentration all died within 7-12 days. Whereas some of those transferred to the control water survived and were cultured for 2 months and grew successfully.

The off springs of 2.2 ppm which were cultured in the same concentration and control water survived and grew at a rate almost equal to those of the control group.

Phenol in the environmental water are highly toxic to aquatic organisms. The international limit for phenol in water as defined by the World Health Organization (W.H.O) has been set at 1 µg/l.¹⁾ Sources of phenol pollution in fresh water include the cooking of coal, chemical plants, gas works and oil refineries, pesticide plants, wood preserving plants, and dye manufacturing plants.²⁻⁶⁾

For a number of fishes the lethal concentration doses of phenol which cause 50% mortalities within 96 h (96 h-LC₅₀) have been determined. These doses differed greatly according to the subjected species. In rainbow trout it was about 4.2-5.0 ppm,⁷⁾ in Goldfish 44.5 ppm,⁸⁾ and in zebrafish 24.9 ppm.⁹⁾

In our previous paper,⁹⁾ we determined that phenol acts directly on the gills and cause hypoxia. Furthermore, it cause physiological damages to the kidney, liver and intestine. In the following study, the chronic toxic effect of phenol on behavioral, histological and hematological changes, spawning, and survival rate of eggs and larvae of

zebrafish were explored. The purpose of the study was attained by culturing of the parental and off-springs of zebrafish in low concentration of phenol. Histological changes of liver, kidney, gill, intestine and ovary due to the toxic doses of phenol for zebrafish *Branchydanio rerio* were studied.

Material and Methods

Experiment 1

Two and half months old zebrafish were bought from a local fish dealer and kept in 30 l aquaria at 24°C. They served as stock fish. They were used after 2 weeks acclimatization to the new environment. Experiment at the following concentrations were carried out in duplicate; control experimental water (0 ppm), 2.2, 4.9, and 24 ppm phenol. Each duplicated concentration named as group A and group B. Each group consisted of 3 females and 6 males and were kept in 15 l aquarium. The fish varried in body weight from 0.25 to 0.31 g and in total body length from 1.3 to 3.3 cm. This experiment was performed for 3

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months.

For the maintainance of constant phenol concentrations during the course of experiment the solutions were renewed once a day. Phenol concentrations were measured by Aminoantipyrine method,¹⁰⁾ prior to and immediately after each renewal. The fish were fed with Tetramine twice a day.

Behavioral changes and time of death of each fish were recorded. Death of the fish was determined by the cessation of gill movements and lack of response to mechanical stimulus.

Interval samplings were carried out on 1 male and 1 female of group A, 1 female and 1 male of group B, respectively after 1 and 2 months and the rest were sacrificed at the termination of the experiment.

Experiment 2

First breeding experiment. Two hundred eggs of the parental fish of the experiment one which were treated with control experimental water (0 ppm), 2.2, and 4.9 ppm phenol concentrations were collected after 15 days treatments. Eggs from each concentration were divided into A and B groups, 100 each, and then exposed to the same phenol concentration as their parents. No fish spawned at 24 ppm. This experiment was performed for two months.

Second breeding experiment. Three hundred eggs of the parental fish of the experiment one which were treated with 2.2 and 4.9 ppm were collected after 30 days of treatments. Eggs of each concentration was divided into A, B and C groups, 100 each. Group A was exposed to the same phenol concentration as the parental fish. Groups B and C were washed twice with control water. Thereafter, group B was replaced in its initial phenol concentration while group C was kept in control water (0 ppm). Control groups were made by collecting 200 eggs of the parents which were kept in the control water of experiment one. These eggs were divided into two groups, 100 each. One group was washed with control water and the other one was not. Both were kept in control water. This experiment was carried out for 2 months.

To verify whether any phenol remained on the membrane of eggs after washing by control water, 10 eggs were washed and checked by Aminoantipyrine method.¹⁰⁾ No phenol was detected.

In the first and second breedings, prior to hatch the phenol solutions were changed every 24 h and

the non-vital eggs were simultaneously separated. From hatch to post-larvae stage, the solutions were changed every 48 h to reduce stress on newly hatched larvae. When all the larvae started to swim freely and were capable of taking artificial foods (after 4 days), the solution was changed every 36 h. After 10 days, the solution was changed every 24 h. In the first 10 days 2 l and thereafter 15 l capacity aquaria were used.

The larvae were fed two days after hatch when the yolk sac had not yet been completely absorbed. The diet was consisted of Tetramine and rainbow trout pellets made into solution and given to them twice a day. In addition boiled egg's yolk was also solved and given once a day. After 15 days, only Tetramine was given twice a day.

In all the experiments dechlorinated tap water was used. Water hardness (measured as CaCO₃), D.O., and pH were 57–61 ppm, 6.4–8.5 ppm, and 6.1–6.5, respectively. The water temperature was controlled thermostatically and kept at 24±1°C. Histologically, the gills, liver, kidney, intestine were stained with hematoxylin and Eosin (H-E), and hematologically the blood cells were stained with May Grünwald Geimsa. The methods of these studies were the same as explained in our previous paper.⁹⁾

Results

Experiment 1

Histological pictures of gill, liver, kidney, intestine, and hematological features of blood cells are shown in Figs. 1–8. Behavioral, histological, and hematological changes of zebra fish treated with phenol are as follows.

A) 24 ppm phenol concentration. During the course of experiment, the phenol concentration ranged from 22 to 26 ppm immediately after renewing and 19 to 22 ppm prior to renewing.

The fish were affected immediately after placing at 24 ppm phenol solution. They lost their balance, swam wildly up and down, and the movements of the mouth and gills opercles were violent. The majority of the fish permanently lost their balances and then death occurred within a few days. In these fish the gill, kidney, liver, intestine and blood cells were severely damaged as were explained in detail in our previous paper.⁹⁾

During this exposure, only 3 out of 9 fish from each duplicated phenol concentration survived. Abnormal behaviors of these fish gradually diminished (visible for about 10 min, only at the time

- Fig. 1.** Gill filaments of the fish killed at 4.9 ppm after 1 month. The epithelial layer is swollen and the epithelial cells are far from the pillar cells. Stained with Hematoxylin-Eosin (H-E), $\times 250$.
- Fig. 2.** Gill filaments of the fish killed by exposure to 24 ppm after 29 h. The epithelial layer is stripped away, only the pillar cells remained. H-E, $\times 250$.
- Fig. 3.** Kidney of the fish killed by exposure to 24 ppm after 48 h. Necrosis of nephrons and agglutination of blood cells has occurred. H-E, $\times 500$.
- Fig. 4.** Liver cells of the fish killed at 4.9 ppm after 1 month. Extravasation of blood vessels has occurred. H-E, $\times 500$.
- Fig. 5.** Intestine of the fish killed at 24 ppm after 3 months. The Lamina propria became scanty and the goblet cells are few in number. H-E, $\times 500$.
- Fig. 6.** Red blood cells of the fish killed by exposure to 24 ppm after 62 h. The cells membranes are partially or completely removed and many ghosts are present. Stained with May-Grunwald-Geimsa, $\times 250$.
- Fig. 7.** Ovary of the fish killed at 4.9 ppm after 2 months. Some oocytes are under atretia. H-E, $\times 250$.
- Fig. 8.** Ovary of the fish killed at 24 ppm after 3 months. Ovary contained only immature oocytes. H-E, $\times 250$.

of renewing the solutions). They did not eat in the first two weeks, though afterwards gradually started to eat. The average body weight was 0.19 at the end of the experiment. No fish spawned during the experiment.

Blood cells were few in number in the capillaries of the liver and kidney of the fish sacrificed at the interval samplings (after 1 or 2 months). Histological damages of the intestine, liver, kidney and gills of the fish in interval samplings were not as acute as those of which died due to phenol in the first few days. The number of disintegrated or pre-disintegrating blood cells were few in number. The last fish of each duplicated group which were killed at the termination showed even better conditions. In these fish, the blood cells inside the capillaries of the liver or kidney were almost identical to those of the controls and the damages to other organs were also slight. At the intervals and final samplings, the ovaries contained only immature oocytes.

B) 4.9 ppm phenol solution. During the course of the experiment, the phenol concentration ranged from 4.2 to 5.2 ppm immediately after renewing and 2.0 to 3.5 ppm prior to renewing.

At 4.9 ppm, the fish showed such abnormal behaviors as wild swimming and violent movements of the gill operculars only after renewing the solution. The abnormal behaviors gradually diminished as the fish recovered to normal. All of them displayed good appetite. The average body weight at the end was 0.29 g. Spawnings under this regime were frequently observed. The spawning intervals ranged between 4–9 days, those were longer than the control group which were ranged between 2–5 days.

Hematologically, some blood cells were abnormal in shape and size, and a few were in either disintegrated or pre-disintegrating stage. Histologically, slight damage to the liver, kidney and gills were observed in the interval samplings (after 1 or 2 months). The blood vessels in both kidney and liver were compacted with blood cells. The epithelial layers of gill filament and secondary gill filament were swollen and the epithelial cells were arranged in outer parts. The intestine did not show any sign of damages for the most parts, though in a few cases the epithelial layer were slightly inflamed. At the termination, the above described damages were greatly recovered. The ovaries contained immature and maturing oocytes, and some of them were under atretia at interval and final samplings.

Table 1. Percentage mortalities of eggs and larvae in control water and phenol solutions (first breeding)

Day	Control		2.2 ppm		4.9 ppm	
	A	B	A	B	A	B
1	4	7	2	3	6	6
2	21	20	32	26	21	32
3	4	3	1	2	3	8
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	4	0	4	0	4	4
7	5	10	6	6	6	7
8	5	6	8	11	21	22
9	10	0	5	6	15	10
10	4	4	4	5	10	6
11	1	0	4	2	7	3
12	2	4	4	3	7	2
13	0	0	0	1	—	—
14	0	0	0	0		
15	0	0	0	0		
Total	60%	54%	70%	65%	100%	100%

A and B are duplicates of the same solution.

C) 2.2 ppm phenol concentration. During the course of the experiment the phenol concentration ranged from 2.0 to 2.5 ppm immediately after renewing and from 0.8 to 1.6 ppm prior to renewing.

Under this regime the fish behaved almost similar to those of the control groups. The fish also showed good appetite. The average body weight at the end of experiment was 0.32 g.

The blood cells showed different shapes and sizes in a few cases however, they were similar to the control in most cases. Histologically, in the interval and final samplings the condition of intestine, liver and kidney were nearly identical to those of the control. The epithelial layers of the gills in a few cases were slightly swollen.

Under this regime, spawnings were observed frequently. The spawnings interval ranged between 4–8 days during the course of experiment. Histologically, the ovaries contained oocytes at various maturation stages and a few of them were under atretia in the interval and final samplings. The atretic oocytes in the ovaries of the fish at 2.2 ppm were quantitatively less than those at 4.9 ppm.

Experiment 2

First breeding experiment. Larvae in the control, 2.2, and 4.9 ppm phenol solutions were hatched after 2–3 days. Eggs and larvae mortalities are shown in Table 1. The mortalities of

Table 2. Percentage mortalities of eggs and larvae in the control and phenol solutions (second breeding)

Day	Control		2.2 ppm			4.9 ppm		
	A	B	A	B	C	A	B	C
1	35	30	40	45	43	60	75	50
2	0	0	0	0	0	0	5	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	20	15	5	15	10	30	10	20
6	10	15	20	5	9	10	5	11
7	0	0	5	2	1	—	5	2
8	0	0	0	0	0	—	—	0
9	0	0	0	0	0	—	—	0
10	0	0	0	0	0	—	—	0
Total	65%	60%	70%	67%	63%	100%	100%	83%

A. Eggs without wash were placed in the same concentration as their parental stock

B. Eggs after wash were placed in the same concentration as their parental stock

C. Eggs after wash were placed in the control water

the off-springs were maximum during the embryonic development and when the larvae commenced active feeding. All the larvae at 4.9 ppm died after 12 days of exposure. At 2.2 ppm, 30% and 35% of the A and B and at control, 40 and 46% of the A and B groups remained alive, respectively. The survived larvae grew successfully till the end of the experiment. The average body weights at 2.2 ppm were 0.26 and 0.29 g for A and B, and at controls they were 0.25 and 0.28 g for A and B groups, respectively.

Second breeding experiment. Eggs and larvae mortalities of control, 2.2, and 4.9 ppm groups are shown in Table 2. Eggs and larvae of A and B groups of 4.9 ppm died within 7 days, while 17% of the larvae at C group survived till the end of the experiment. At 2.2 ppm; 30% of A, 33% of B, and 37% of C group remained alive till the termination of the experiment. At the control experiment, 35% of A and 40% of B group survived. These larvae showed good appetite almost comparable to those of the control ones and their growth was successful.

The average body weight of C group at 4.9 ppm was 0.21 g. At 2.2 ppm, the average body weights were 0.29, 0.31, and 0.30 g for A, B, and C groups, and at control groups they were 0.31, and 0.30 g for A and B groups, respectively.

Histologically, the liver, kidney, intestine, gills, and hematologically the blood cells of the survived larvae were almost identical to those of the control groups in the first and second breedings.

Discussion

Zebrafish reacted to 2.2 and 4.9 ppm phenol almost immediately after placement in these solutions. They swam wildly up and down, the movement of the mouth and gill opercles were violent, the fish tried to keep themselves near the surface of the water and gulped air. The abnormal behaviors at 2.2 ppm were more severe and lasted longer than those at 4.9 ppm. These behaviors occurred due to the toxic effect of phenol as were stated in the acute tests.⁹⁾ However, the fish behaved almost similarly to the control ones when were kept at 2.2 ppm.

Histologically, the fish exposed to the 2.2 ppm showed severe damages to the gills, liver, kidney and intestine, and most fish died at 2.2 ppm within few days. However, the extent of damages of these organs were slight at 4.9 ppm and no fish died. As the degree of damages in the dead fish were greater than those in the survived fish, it may be stated that the cause of death is due to these damages, most possibly due to asphyxia. Furthermore, the physiological changes of the liver and kidney could be also the cause of death as were stated in the acute tests.⁹⁾ At 2.2 ppm, there were not found any clear destructive histological changes in the gills, intestine, liver and kidney. It is suggested that the concentration of 2.2 ppm is too low to cause damages to these organs.

At 2.2 ppm, 3 out of 9 fish of each duplicated group remained alive. These fish did not eat in the first 2 weeks though gradually commenced eating and till termination of the experiment the histological damages were greatly improved. The

fish at 4.9 ppm also showed better apparent and histological conditions at the termination as compared with the interval samplings. These phenomena may be due to the adaptation of the fish to the phenol toxicity. Adaptation presumably is related to the improvement of the liver in the process of detoxification of phenol as well as improvement of the kidney for removal of wastes from the body. Detoxification role of the liver has been described in other animals.¹¹⁻¹⁴⁾ Since the number of disintegrated and deformed blood cells were not so distinctive at the end of the experiment, it might also indicate that the defensive reaction improved over the course of the phenol treatments and acted effectively against the toxic influence of phenol in zebrafish.

Spawning intervals of the parents at 4.9 and 2.2 ppm phenol solution were apt to be longer as compared to the control. The ovaries of 4.9 and 2.2 ppm consisted oocytes in various maturational stages. Some of these oocytes were under atretia which their quantities were distinctly greater at 4.9 ppm than at 2.2 ppm. All the fish at 24 ppm possessed only immature oocytes and no one spawned during the experiment. From these results it is obvious that the phenol toxicity is an essential cause of partial or total prevention of gonadal maturation in zebrafish.

In the first breeding, the off-springs (eggs and larvae) of 4.9 ppm groups perished within 12 days (Table 1), though in the second breeding the off-springs died within 7 days (Table 2). It might suggested that the longer exposure of parents to the same phenol concentration causes reduction of the eggs qualities.

In the second breeding, the mortalities of the off springs of 4.9 ppm were more than those of 2.2 ppm when both groups were cultured at the control water (compare C groups of Table 2). These results indicate that the treatment of parental fish with higher phenol concentration cause more reduction of the eggs quantities.

In zebrafish, the least resistance to phenol was during embryonic development. In addition, actively feeding larvae were more sensitive than the

yolk sac larvae. Similar reactions were observed in the bream.¹⁵⁾

At 2.2 ppm, the cultures of eggs were successful. No distinct differences were observed between the behavior and histological pictures of the larvae which cultured at 2.2 ppm and those of the control. Thus, the minimum concentration of phenol which cause damages to the off-springs survival rates exist between 2.2 and 4.9 ppm.

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