Research Article

The effects of the water-soluble fraction of diesel (WSFD) on behavioral, biochemical, and growth response in common carp (*Cyprinus carpio*)

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Abstract

This study investigated the effect of the water-soluble fraction of diesel (WSFD) on common carp behavioral, biochemical, and growth responses. Fish were divided into four groups, including control group (G1) and three exposures of 4% (G2), 8% (G3), and 16% (G4) WSFD. After 168 hours, weight gain (WG), specific growth rate (SGR), and daily growth rate (DGR) were calculated, and blood samples were taken. The results showed a significant difference between the growth indices of the G1 and the exposure groups ($p<0.05$). The mean of WG, SGR, and DGR indices (7.00 g, 1.85 %, 0.99, respectively) in the G2 had a significant difference with the G3 (4.44 g, 1.12 %, 0.64, respectively) and the G4 (0.89 g, 0.25 %, 0.12, respectively) ($p<0.05$). In addition, there was a significant difference between growth indices in the G3 and the G4 ($p<0.05$). There was a significant difference between the mean cortisol and serum glucose levels of the G1 (3.18 µg/dl, 61.33 mg/dl) and the G4 (10.70 µg/dl /ml, 108.33 mg/dl) ($p<0.05$). Swimming pattern changes, activity level, food intake, and gill movements occurred due to WSFD exposure. The fish behavior was expected in the G1 and G2. However, with increasing the concentration of the WSFD to 16%, an imbalance was observed with swimming changes, loss of appetite, decreased mobility, and impaired breathing. The present study's findings showed that WSFD exposure causes growth retardation, disturbance of biochemical blood factors, and behavioral changes in fish.

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Introduction

Different kinds of natural polluting substances and some human activities can pollute aquatic environments. Fish are one of the essential creatures living in aquatic environments. Therefore, pollution of water resources causes various damages such as stress, growth retardation, susceptibility to various diseases, pathological lesions, and even fish death (Nogueira *et al*., 2011; Sharifpour *et al*., 2011). Among different types of pollutants, oil and its products are the most common pollutants in aquatic environments due to their numerous applications and high consumption (Collier *et al*., 2013). The amount of oil and its products' entrance into water resources is estimated at 1300000 metric tons in a year (Rodrigues *et al*., 2010). Diesel is one of the derivatives of crude oil used in a large volume daily as the fuel for energy production (Santos *et al*., 2013). The highest percentage of diesel components are hydrocarbons (Khatun *et al*., 2021); In addition to these materials, there are some amounts of heavy metals (iron, nickel, copper), nitrogen, sulfur, and preservatives in the structure of diesel (Delunardo *et al*., 2020). Part of the diesel dissolves in aquatic environments during leakage, and part of it remains insoluble on the water's surface. The WSFD is absorbed 10 to 100 times more than the insoluble part of diesel into body fish (Vanzella *et al*., 2007). Monocyclic (BTX) and polycyclic (PAHs) hydrocarbons in diesel dissolve quickly in water due to their low molecular weight (Santos *et al*., 2013). The soluble phase of diesel is highly toxic to fish (Akaishi *et al*., 2004). So that after absorption (skin, gastrointestinal tract, and gills) by fish, a large volume of free radicals is produced due to the metabolism of aromatic hydrocarbons, which destroy cells and impair the function of a living creature (Simonato *et al*., 2008). Aromatic hydrocarbons in the diesel solution phase are very lipophilic, and their accumulation in various body tissues causes dysfunction of the respiratory system, osmotic balance, weakening of the immune system, growth retardation, and genetic mutations (Claireaux and Davpdi, 2010; Rodrigues *et al*., 2010; Delunardo *et al*., 2013; Cherr *et al*., 2017; Freire *et al*., 2020).

The fish behavior is affected by the body's normal function and the aquatic environment's condition (Lari *et al*., 2016). The diesel entrance into the water sources changes the quality and condition of the water and causes stress to the fish (Kochhann *et al*., 2015). On the other hand, the absorption of the toxin in the WSFD by fish causes various damages in vital organs that disrupt the body's normal function and causes abnormal behaviors in fish (Kasumyan, 2001). Studying and

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identifying behavioral changes while exposed to the diesel solution phase is very important for rapid detection of water pollution to prevent the progression of damage to the fish. Pathological lesions due to exposure to the diesel solution phase in vital organs such as the liver, kidney, and gill disrupt the metabolic cycle in the body and reduce fish growth (Esenowo and Ugwamba, 2010; Olyaei *et al*., 2014). So growth indices are a reliable factor for assessing the fish vulnerability while exposed to the diesel solution phase (Gusmao *et al*., 2012). Blood serum biochemical factors are considered an essential criterion for assessing the physiological status of fish (Jahanbakhshi and Hedayati, 2013). Biochemical parameter changes indicate fish's reaction to environmental changes such as water pollution (Eriegha *et al*., 2017). Two parameters of cortisol and glucose can be used to determine the effect of the WSFD on fish. Common carp is one of the most important economic species in the carp family. Carp is the largest family of freshwater fish. The main habitat of these species is in Asia, but it has been introduced for breeding in most parts of the world and is the second most crucial breeding species (Hameed and Al-Azawi, 2016). One of the most significant financial species in the Caspian Sea and Iranian fish farms is common carp being popular among Iranian people (Sobhan Ardakani and Jafari, 2015).

This study aimed to investigate the effect of the WSFD on behavioral changes, growth indices, and biochemical factors in common carp.

Materials and Methods

Research design

A place with dimensions of 50 square meters was considered for the project. The principles of biosafety were observed within the research site to prevent any contaminations during the research period and inspected daily. Two aquariums with a capacity of 500 liters were used for quarantine, and 12 aquariums with a volume of 200 L were used for fish diesel solution-phase exposure. Three plastic baths with a capacity of 600 L were also prepared to mix diesel with water. Dechlorinated tap water was used, and the aquariums were aerated with an air stone connected to a central air pump. The length of the light period was programmed as 12 hours of light and 12 hours of darkness. An electric thermometer was consumed to measure the water temperature, and standard freshwater aquarium special tests were used to assess the pH and oxygen dissolved in the water. The duration of the study was 21 days.

Fish

Ninety-six common carp weighing 50±0.50 g and 16±0.50 cm were bought from the carp aquaculture center Sangar, Gilan province. After transferring the fish to the research site, they were bathed with

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2% sea salt for 30 minutes to ensure the elimination of pathogens. Then, they were divided into two groups and released in quarantine aquariums for 14 days. During the quarantine period, water was adjusted to 22 ± 2 °C, pH 7.6, and oxygen 7.00 ± 0.20 mg L^{-1}. Feeding was done two times a day, in the morning and evening, based on 12% biomass of a commercial feed (Carp growing stage EX-CG Beyza 21 Feed Mill Company). Freshwater that had all the physical and chemical parameters of quarantine aquarium water was replaced based on reduced water volume.

Extraction of diesel solution phase

Two hundred L current diesel was bought from petroleum products distribution centers. Diesel was mixed with water to extract the solution phase of diesel by the Anderson *et al*. (1974) method. The diesel was mixed with water to obtain proportionally selected density and placed in a dark place for 23 h. Then it was gently mixed with an electric mixer during this time. The mixture of diesel and water was then allowed to remain for 1 h. The mix of diesel and water was then exposed to direct sunlight for 5 h and was mixed with an electric mixer. Finally, the water and diesel mixture was allowed to remain for 1 h at which the soluble phase to be separated from the insoluble one. The whole solution phase was carefully separated from the bottom of the mixture container by siphoning and transferred to the specific aquariums. After the mixing process, the solution phase was separated from the insoluble part and transferred to the exposed aquariums according to the determined densities.

Exposure to the diesel solution-phase test

Experimental groups were divided into four groups, including one control group (G1) without a water-soluble fraction of diesel (WSFD) and three groups of WSFD with 4% (G2), 8% (G3), and 16% (G4) densities based on the amount of LC_{50} (640±30) of WSFD that was determined by Rodrigues *et al*. (2010). Each group had triplicates with eight fish. The fish were exposed to the diesel solution phase for 168 h. Water temperature conditions were similar to the quarantine tank during the exposure period, and the light and dark periods were 12 and 12 h. Feeding was done two times in the morning and evening daily. Moreover, at the end of the lightning period, the leftover food and waste were siphoned, and water that had a similar condition to control or exposed groups was added to the aquarium.

Analysis of growth parameters

The fish were weighed with digital scales before the start of WSFD exposure to evaluate the growth indices, and at the end of the exposure, total length and fork length were measured and recorded with a ruler. The average weight, specific growth rate, daily growth rate, and survival percentage rate were calculated (Eserowo and Ugwamba, 2010).

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Weight gain = W2 (g) - W1 (g)

Specific growth rate (SGR) = 100 × (ln W2 - ln W1) / T

Daily growth rate (DGR) = (W2 - W1) / T

Survival rate (%) = 100 × (final number of fish / initial number of fish)

Where W1 is the initial weight, W2 is the final weight, T is the number of days in the feeding period, and ln is the Natural logarithm.

Behavior changes

The experimental groups of fish were observed every 12 h for abnormal behaviors such as erratic swimming, decrease or increase of the gill movement, food intake, and activity to examine the behavioral changes during 168 h of WSFD exposure. The exposure elapsed time and the experimental groups were recorded by film or photo as soon as any behavior change in individual fish or group was observed. Furthermore, the groups were evaluated and compared together at the end of the exposure period.

Biochemical indicators

After 168 h of WSFD exposure, six species were randomly selected from each group, and after fish anesthesia, blood was taken from caudal vein with a heparin-soaked syringe. The blood was transferred to 1.5 cc micro tubes and placed in a centrifuge at 3000 rpm for 5 minutes for serum separation. The serums were packaged next to an ice pack, sent to a laboratory and stored in a -20 freezer until biochemical tests were performed to be evaluated their glucose and cortisol (Hedayati, 2018).

Data analysis

The variance test (ANOVA) was used to evaluate growth parameters and biochemical indicators. If there was a significant difference, it was analyzed with the Tukey test to compare the means at a significant level of 0.05. The software Spss.v.26 was used.

Results

Growth indicators

According to Table 1, the results showed that the mean weight gain, specific growth rate, daily growth, and survival percentage were significantly different between the control and WSFD groups ($p<0.05$). There was a significant difference between the mean weight gain in the G2 (7.00) with G3 (4.44) and the G4 (0.89) ($p<0.05$). Also, there was a significant difference between the mean weight gain in the G3 (4.44) and the G4 (0.89) ($p<0.05$). There was a significant difference between the mean specific growth rate in the G2 (1.85) with G3 (1.12) and G4 (0.25) ($p<0.05$). Also, there was a significant difference between the mean specific growth rate in the G3 (1.12) and the G4 (0.25) ($p<0.05$). There was a significant difference between the mean daily growth in the G2 (0.99) with the G3 (0.64) and the G4 (0.12) ($p<0.05$). Also, there was a significant difference between the mean daily growth in the G3

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(0.64) and the G4 (0.12) ($p<0.05$). There was no significant difference in survival rate between the control and exposure groups ($p>0.05$). However, in the G4, the survival rate (88.88%) decreases compared to the other experimental groups (100%).

Table 1. Effect of water-soluble fraction diesel on weight gain, SGR, mean daily growth, and Survival of common carp

Indicators	Groups			
	G1	G2	G3	G4
Initial weight(g)	50.22 ± 0.44^a	50.22 ± 0.44^a	50.44 ± 0.52^a	50.33 ± 0.50^a
Final weight(g)	58.55 ± 1.01^a	57.22 ± 0.66^a	54.89 ± 0.78^b	51.00 ± 1.11^c
Initial length(cm)	16.22 ± 0.44^a	16.33 ± 0.50^a	16.22 ± 0.44^a	16.22 ± 0.44^a
Final length(cm)	16.70 ± 0.41^a	16.51 ± 0.45^a	16.32 ± 0.41^a	16.26 ± 0.41^a
Weight gain(g)	8.33 ± 0.70^a	7.00 ± 0.86^a	4.44 ± 0.29^b	0.89 ± 0.96^c
Specific growth rate (%)	2.17 ± 0.13^a	1.85 ± 0.24^a	1.12 ± 0.25^b	0.25 ± 0.26^c
Mean daily growth	1.18 ± 0.09^a	0.99 ± 0.12^a	0.64 ± 0.11^b	0.12 ± 0.13^c
Survival percentage (%)	100^a	100^a	100^a	88.88^a

Statistical differences are shown by different letters ($p<0.05$).

Biochemical factors

According to Table 2, the mean of cortisol in the G1 (3.18 µg/dl) was significantly different from the G4 (10.70 µg/dl) (P=0.038). There was no significant difference between these groups ($p>0.05$), despite the increase in mean cortisol in the G2 (4.30 µg/dl) and the G3 (6.93 µg/dl) groups compared to the G1. The cortisol means in exposed groups of fish did not show any difference ($p>0.05$).

The results of Table 2 showed that the mean blood plasma glucose in the G1 (61.33 mg/dl) was significantly different from the G4 (108.33 mg/dl) (p=0.038). There was no significant difference between these groups ($p>0.05$), despite the increase in mean blood plasma glucose in the G2 (76.33 mg/dl) and G3 (94.00 mg/dl) groups compared to the G1. The mean blood plasma glucose in the exposed group of fish did not differ ($p>0.05$).

Table 2. Effect of water-soluble fraction diesel on the blood serum biochemical indices of common carp

Indicators	Groups			
	G1	G2	G3	G4
Cortisol (µg/dl)	3.18 ± 1.31^a	4.30 ± 1.60^a	6.93 ± 1.82^a	10.70 ± 1.92^b
Glucose (mg/dl)	61.33 ± 14.43^a	76.33 ± 7.21^a	94.00 ± 6.65^a	108.33 ± 2.60^b

Statistical differences are shown by different letters ($p<0.05$).

Behavioral changes

Behavioral changes examination in Table 3 showed that WSFD exposure hurt behavior patterns. The fish activity was regular in G1 and G2 so that they reacted quickly to external stimuli. They swam in the water column, and any abnormal

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changes in the swimming pattern were not observed. The fish were immediately present at the spill site during feeding and searching for food on the bottom of the aquarium during the light period. Gill's movement was normal, and there were no signs of breathing problems. In the G3, gill movement increased after 48h of diesel solution-phase exposure. Fish had a slower response to external stimuli than the G1 and G2. Decreased appetite was another behavioral change that made them less interested in eating. Searching for food at the bottom of the aquarium was less during the day. After 96 h of exposure, they mostly swam on the surface of the water. Activity and gill movement decreased, sometimes imbalanced, and swimming on the side was observed. The most behavior changes were reported in the G4 compared to other experimental groups so that after 48 h, gill movement extremely increased, mostly swam on the water surface, and gasping was observed. Imbalance in swimming, reduced food intake, and activity were other unusual behaviors. Increasing the time of diesel solution-phase exposure caused lower activity, so a reaction to external stimuli was not observed. After the initial increase in gill movement at the end of the exposure period, the gill movement was languid. Fish's appetite was lost completely. The imbalance was observed with side and sometimes rotational swimming.

Table 3. Effect of water-soluble fraction diesel on the behavior of common carp

Abnormality	Groups			
	G1	G2	G3	G4
Erratic swimming	-	-	+	+++
Rate of reduced	-	-	++	+++
Rate of activity	-	+	++	+++
Gill movement	-	-	++	+++
Loss of balance	-	-	+	++

(_) indicates absent, (+) less frequent, (++) moderately frequent, (+++) highly frequent.

Discussion

Diesel is a crude oil derivative that has high toxicity to fish due to its toxic compounds such as aromatic hydrocarbons, heavy metals, and a percentage of sulfur (Delunardo *et al*., 2020). Pollution possibility of water resources has become more than before because worldwide consumption of this product has increased. Diesel can cause other damages such as abnormal behaviors, growth index disorders, disruption of biochemical blood conditions, and even fish death (Simonato *et al*., 2008; Hameed and Al-Azawi, 2016; Khatun *et al*., 2021). With due attention to diesel's different formulation and chemical composition in other geographic areas, it is crucial to investigate the fish WSFD exposure damages in different countries. This

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study exposed common carp to sub-lethal densities of WSFD for 168 hours. After examining behavioral changes, assessing cortisol and glucose levels of blood serum, and growth factor estimation, the data were statistically analyzed. The results showed a difference between exposed and non-exposed groups to the diesel solution. There were also differences between the exposed groups.

Growth and survival percentage indices showed the fish's optimal health status (Anwar *et al*., 2020). Therefore, the study of growth indices changes can indicate the fish damage occurrence while WSFD exposure. In addition to environmental changes and stressful conditions to fish after diesel leakage into the water, the diesel solution-phase's toxic compounds can disrupt the body's physiological and biochemical conditions and create pathologic lesions in vital organs, causing functional disorders (Collier *et al*., 2013). These changes reduce the fish's appetite, and the food intake is less than average. Also, increased energy consumption for detoxification causes growth retardation (Sandrini-Neto *et al*., 2016). Nwabueze and Agbogidi (2010), in the effect of 0, 12.5, 25, 50, and 100 crude oil solution-phase densities on growth indices on *Heterobranchus bidorsalis* for ten weeks' study, concluded a significant difference between the mean weight of the control group (7.64) and the mean weight of the exposed groups 12.5% (3.47), 25% (1.47), 50% (-0.11), and 100% (-0.26) ($p<0.05$). They also reported that the increase of crude oil solution-phase density in the water caused the reduction of mean weight. The results of their study were consistent with the results of the present study, so there was a significant difference between the mean weight of WSFD exposed groups and the control group ($p<0.05$). The increase in WSFD caused a lower average weight gain. The highest mean weight after the control group (8.33) was in the 4% group (7.00), and the lowest mean weight gain was in the 16% group (0.89). Another study investigated the effect of oil solution-phase on growth indices of *Odontesthes argentinensis* fish larvae; the results showed a significant difference between the specific growth rate percentage in exposed to oil solution-phase and non-exposed groups ($p<0.05$). As the concentration of the oil-soluble phase in water increased, the specific growth rate decreased, so the mean specific rate percentage in the 20% oil-soluble phase group (5.71) was significantly different from other experimental groups ($p<0.05$) (Gusmao *et al*., 2012). The present study results also showed a significant difference between the groups exposed to the WSFD and the control group ($p<0.05$). Also, a significant difference was observed between the groups exposed to the WSFD ($p<0.05$). The lowest mean specific growth rate percentage was in the 16% group (0.25), and the highest mean specific growth rate percentage was in the 4% group (1.85). Therefore, as the WSFD increased, the specific rate percentage increased less than normal. In this study, a significant difference was reported between the control group's mean daily growth rate percentage and the exposed groups to the WSFD ($p<0.05$). There was also a significant difference between the mean daily growth percentage of the exposed groups ($p<0.05$).

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This study's results confirmed Anwar *et al.* (2020), who stated that the crude oil solution-phase affected the growth indices, and the increase in the concentration of crude oil solution-phase caused the intensity of these changes to increase. Dede and Kaglo (2001) studied the effect of the WSFD on the survival rate of *O. niloticus* larvae. Their studies showed a significant difference between the survival rate percentage of the experimental groups after 96 h of 0, 1.6, 3.2, 6.4, 12.8, and 19.2 ppm diesel solution-phase densities exposure ($p<0.05$). So the survival rate percentage in the control group was 100, and in the 19.2 density of diesel solution-phase exposed group was zero. These results are consistent with the present study's results which state that the diesel solution-phase density increase causes the survival rate reduction.

Examination of blood serum biochemical parameters gives a complete report on the fish's health status (Sabouri *et al*., 2017). Blood biochemical parameter changes can be caused by a wide range of factors such as environmental changes, water pollution, the presence of pathogens, poisoning, and nutritional problems. (Kochhan *et al*., 2015; Rezende *et al*., 2016 Eriegha *et al*., 2017). Another study was performed on the *Prochilodus lineatus* larvae exposed to the WSFD; the serum glucose level increased after 96 h of exposure and was significantly different from the control group ($p<0.05$) (Simonato *et al*., 2008). The present study results were consistent with Simonato *et al.* (2008). The mean blood serum glucose level in the 4% group (76.33), 8% (94.00), and 16% (108.33) WSFD increased compared to the mean blood serum glucose level in the control group (61.33). There was a significant difference between the mean blood serum glucose levels of the 16% WSFD group and the control group ($p<0.05$). Eriegha *et al.* (2017) examined the effect of 28, 41, 55, and 69 mg/l crude oil solution-phase densities on the mean blood serum glucose level of *O. niloticus* larvae. Their study's results showed a significant difference between the control group and the exposure groups after 84 days of exposure to different crude oil solution-phase densities ($p<0.05$). They also stated that by increasing the concentration of the crude oil solution phase, the average glucose level in the blood serum increased. There was a significant difference between the mean blood serum glucose level of the 69 mg/l (255.67) and 55 mg/l (237.67) exposed groups densities, and the 41 mg/l (217.33) and 28 mg/l (216.67) exposed groups densities ($p<0.05$). These results were consistent with the present study stating that the WSFD caused blood glucose level changes and that these changes become more severe with diesel solution-phase density increase. The most abundant corticosteroid in the fish's blood is cortisol. HPA (The Hypothalamic, Pituitary, Adrenal) pathway is activated when stressful conditions occur, increasing blood serum cortisol levels (Pacheco and Santos, 2001). A study by Jahanbakhshi *et al.* (2014) examined the effect of crude oil exposure on blood serum cortisol levels in common carp; the results showed that after 96 h of 22.4±0.03 ppm crude oil density exposure, blood serum cortisol levels increased significantly ($p<0.05$). The

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present study results were consistent with the Jahanbakhshi *et al.* (2014). So that the mean blood serum cortisol levels in the 4% (4.30), 8% (6.93), and 16% (10.70) WSFD groups increased compared to the mean blood serum cortisol levels of the control group (3.18). There was also a significant difference between the mean blood serum cortisol level of the 16% diesel solution-phase group and the control group ($p<0.05$). In another study, Simonato *et al.* (2008) investigated the effect of WSFD on the mean blood serum cortisol level in *Prochilodus lineatus*. The results showed that the mean blood serum cortisol level increased after exposure from 24 to 48 h. However, the mean blood serum cortisol levels decreased significantly 15 days after WSFD exposure ($p<0.05$). This decrease was due to the damage to various organs, such as the kidney disrupting the HPA pathway by contaminant density increase, amounts of structural, chemical compounds, and exposure duration (Pacheco and Santos, 2001). After 120 h of the Caspian kutum (*Rutilus kutum*) exposure to the crude oil solution phase, the mean blood serum cortisol level increased. Therefore, there was a significant difference in blood serum cortisol levels of crude oil exposed groups and the control group ($p<0.05$) (Sabouri *et al*., 2017). Sabouri *et al.* (2017) results were consistent with the present study results that WSFD exposure increased the mean blood serum cortisol levels.

The fish behavior indicates the response to the internal (physiological) and external (environmental) factors. Fish tells us about its physical condition by its behavior. Therefore, examining behavior changes can provide good information about the fish's health status (Gerhardt, 2007). In a study of 96 h diesel oil suspension exposure of three species of Indian carps (Ruha, Calta, and Merigal), the results showed all three types of swimming patterns, rate of activity, food intake, and gill movements changed, and the severity of the lesions increased over time, and diesel oil suspension density increase. So they reported more severe abnormalities than other experimental groups at 0.75 ppm density after 96 h (Khatun *et al*., 2021). The results were consistent with the present study stating WSFD exposure caused changes in swimming pattern, food intake, rate of activity, loss of balance and orientation, increase or decrease in gill movements, and the intensity of behavior changes increased by increasing the diesel solution-phase density. Hameed and Al-Azawi (2016) studied the effect of WSFD on the common carp behavior changes. The results showed a significant difference between the control group and the WSFD exposed groups. In the control group, any behavior abnormalities were not observed. Nevertheless, the exposed groups showed the swimming pattern change, gill movement, rate of activity, and loss of balance and orientation. The most severe behavioral abnormalities were in the highest diesel solution-phase density among all exposed groups. In the 0.007% WSFD group, proximity to the water surface and gill movement increased. However, in the 0.01% WSFD group, loss of balance, gill movement decrease, fish activity decrease, and spiral swimming was reported.

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These results were consistent with the results of the present study.

WSFD made extensive changes in the level of blood biochemical factors (glucose and cortisol), disruption of normal growth (growth indices reduction) and behavioral changes in fish. The combination of these injuries endangered the fish health and even led to death. As the WSFD density and duration exposure increased, the intensity of changes also increased. So, it is important to monitor the sources that can leak diesel into the aquatic environments and prevent the following pollution. Also, providing diagnostic methods of poisoning can be effective in identification and prevention the damage and elimination the sources of contaminants, and consequently leads to reduce the economic damage caused by water pollution with diesel.

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Conflict of interest

Authors have no conflict of interest on this work.

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