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Determination of the Acute and Chronic Toxicity of Sulfate from the Sulfur Autotrophic Denitrification Process to Juvenile Zebrafish (*Danio rerio*)

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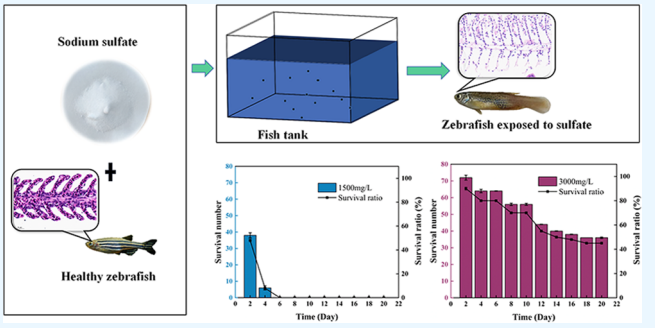
ABSTRACT: Sulfur-based materials are widely used as electron donors for denitrification to enhance nitrogen removal from water. This leads to an increased sulfate concentration in the effluent or sulfate accumulation in recirculating aquaculture systems. This study explored acute and chronic toxicity of sulfate to juvenile zebrafish (*Danio rerio*) and investigated the histopathological changes in the gills of juvenile zebrafish exposed to sulfate. Results show that zebrafish had a high tolerance to sulfate, with no acute toxicity at sulfate concentrations from 250 to 3200 mg/L. For the chronic toxicity study, it was found that zebrafish mortality decreased with the increase in sulfate concentrations ranging from 250 to 1500 mg/L. In contrast, when the sulfate concentration was 1500−3000 mg/L, zebrafish mortality increased with the increasing sulfate concentration. In addition, in the ion balance test, KCl was added to balance the effects of Na^+ from the Na_2SO_4 used to obtain the desired sulfate concentrations, showing that fish mortality correspondingly increased with increasing KCl addition. Furthermore, when living in an environment with elevated sulfate concentrations for a long period, changes were observed in the morphology, behavior, and gill tissue of the zebrafish, including slow and lateral swimming; bottom settling; and large opening and closing, lamellar fusion, and necrosis of gills. This research reveals the toxicity of sulfate to aquatic organisms, providing a scientific basis for the promotion and application of sulfur or sulfur-based materials in autotrophic reduction processes for wastewater treatment.



1. INTRODUCTION

Sulfate, an anion, occurs naturally in the aquatic environment. The sulfate level in freshwater environments is usually low. Most lakes and rivers contain about 20 mg/L sulfate, while seawater contains approximately 3000 mg/L.[1] The cause of sulfate in water is generally divided into natural sources and manmade sources. Natural sources include sulfate released from mineral components, the oxidation of metal sulfides, volcanic eruptions, acid rain, seawater intrusion, and other sources.[2,3] Nowadays, man-made sources are the main cause of sulfate in water and include sulfate-rich wastewater and waste discharged from industries such as mining, metallurgy, food, medicine, smelting, steel manufacturing, kraft pulp and paper mills.[4] Sulfate is stable in water, which makes sulfate wastewater not as easily purified by the natural environment as some other types of wastewater, and the pollution effect easily accumulates.

Recirculating aquaculture systems (RASs) are a culture mode to achieve high-density intensification by treating and recycling water.[5] RAS has been attracting considerable interest because they can alleviate the problems of land use, water use, and wastewater discharge caused by the expansion of aquaculture. However, there is a problem of residual and accumulated nitrogen compounds in RAS. The excessive accumulation of nitrogen compounds will affect the physiological, morphological, and behavioral changes of fish and even lead to fish death.[6−9] In order to remove nitrogen compounds in the RAS, the denitrification unit is established in the RAS to transform nitrate to nitrogen gas. In our previous study, sulfate continuously accumulated in a marine RAS.[10] The observed sulfate accumulation was due to the application of biological elemental sulfur (S^0)-based autotrophic denitrification (SAD) for nitrate removal from the RAS.[10] The disadvantage of SAD is sulfate generation, with 7.54 mg of sulfate formed for each mg of nitrate-N (NO_3^--N) reduced.[11] Therefore, the sulfate concentration in the RAS increased from an initial ∼1300 to 2300 mg/L after 146 days of operation. An increase in the

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Table 1. Mean Values of the Water Quality Variables Monitored during the Acute Sulfate Toxicity to the Zebrafish Experiment

variables	control	200	400	800	1600	3200	recommended values
temperature (°C)	28.91	28.94	28.89	29.00	29.05	29.12	20−30
dissolved oxygen (mg/L)	7.24	7.21	7.16	7.09	7.04	6.99	>5
pH	8.56	8.58	8.58	8.56	8.56	8.56	6−9
alkalinity (mg $CaCO_3$/L)	190	176	195	195	227	205	50−450
hardness (mg $CaCO_3$/L)	226	225	218	229	231	226	50−450
ammonia (mg N/L)	BDL[a]	BDL	BDL	BDL	BDL	BDL	<2
nitrite (mg N/L)	BDL	0.10	0.07	0.06	0.03	0.09	<0.2
nitrate (mg N/L)	0.32	2.35	1.17	1.89	1.58	2.24	<3

[a]BDL: below detection limit.

Table 2. Mortality of Acute Toxicity at Various Sulfate Concentrations

	mortality (%)					
time	control	200 mg/L	400 mg/L	800 mg/L	1600 mg/L	3200 mg/L
24 h	10	5	5	5	0	0
48 h	15	10	10	15	5	0
72 h	25	15	20	25	5	5
96 h	35	25	30	35	10	10

mortality and swimming anomaly was observed when fish lived in water containing a high sulfate content (~2300 mg/L) for a long period (~120 days).[12] The increased sulfate concentration might be a cause of the higher fish mortality.

Sulfate toxicity to aquatic organisms has been investigated in the freshwater environments.[13] Various species have different health limits. The half maximal effective concentration (EC_{50}) for a cladoceran (*Ceriodaphnia dubia*) was 2242−2441 mg sulfate/L, and the 96 h median lethal concentrations (LC_{50}) for larval fathead minnow and a midge (*Creontiades dilutus*) were 4833 and 5992 mg sulfate /L, respectively.[4] As far as fish are concerned, blackhead minnow are sensitive to chronic sulfate exposure, while rainbow trout are not (EC20 > 3240 mg/L).[4] Karjalainen et al. investigated the toxicity of sulfate in humic, soft freshwater to whitefish (*Coregonus lavaretus*) and found that the LC_{50} values of sulfate during the early embryonic period and for the entire embryonic and larval period were 1413 and 1161 mg/L, respectively.[13]

However, in the previous studies addressing sulfate toxicity to aquatic organisms, most of the data on sulfate toxicity were from acute or short-term studies on aquatic invertebrates,[14] lacking chronic toxicity studies. Some toxicities are not immediately fatal or acute, but they may lead to long-term effects, such as disruption of normal behavior and tissue damage.[15] Therefore, it was hard to conclude that a high sulfate concentration led to the high mortality of the zebrafish in our previous RAS study. Chronic toxicity is a sensitive index used to study the sublethal effect of species, which is helpful for determining the minimum concentration of water quality parameters that will have a significant negative effect.[16] Therefore, it is essential to conduct further research on the chronic toxicity of sulfate to fish. In addition, understanding the toxicity of sulfate to fish is a key issue in the popularization and application of SAD technologies for nitrate removal from water.

Therefore, this study was carried out to investigate the influence of sulfate on fish health to promote the application SAD in RAS or wastewater treatment. As an accepted model in toxicologic research,[17] zebrafish (*Danio rerio*) was selected as the test organism in this study. The objectives were to (1) reveal the acute and chronic toxicity of sulfate on zebrafish; (2) investigate the effects of sulfate exposure on the growth performance (i.e., growth and swimming behavior and pathological changes); and (3) observe histopathological alterations in the gills of the fish and reveal the toxicity of sulfate to aquatic organisms.

2. RESULTS AND DISCUSSION

2.1. Acute Sulfate Toxicity to the Zebrafish. As listed in Table 1, the temperature, DO, and pH met the recommended water quality requirements for zebrafish growth. The change in salinity was caused by the compound used to prepare the sulfate storage solution (Na_2SO_4).

During the experimental period, zebrafish exposed to sulfate concentrations of 0, 200, 400, 800, 1600, and 3200 mg/L showed varying mortality at 24, 48, 72, and 96 h (Table 2). The maximum mortality of zebrafish in this experiment was 35% and less than 50%, and it can be concluded that there was no acute toxicity of sulfate to zebrafish from 0 to 3200 mg/L. According to the standard "Water quality-Determination of the acute toxicity of substance to freshwater fish (*Brachydanio rerio* Hamilton-Buchanan)", the mortality of the control group should not exceed 10%, and the abnormal rate of appearance and behavior of the fish in the control group during the experiment should not exceed 10%. In this study, the 96 h mortality of the control was 35%, and there was no correlation between the sulfate concentration and zebrafish mortality within the experimental time (ANOVA, $p > 0.05$). Therefore, it was impossible to calculate the median lethal concentration (LC_{50}) of the zebrafish. However, Karjalainen et al. conducted acute toxicity studies for the early life stages of the European whitefish and found that the LC_{50} values of sulfate for fertilization, the early embryonic period, and the entire embryonic and larval period were 2280, 1413, and 1161 mg/L, respectively.[13] These studies were conducted during the early stages of fish life, such as fertilization, embryo, incubation, and a few days after incubation, which were more sensitive to sulfate.[14] The zebrafish used in this study were in the juvenile stage, which is the probable reason for the inconsistent experimental results. The difference in acute toxicity might be due to the change in water quality and tested species. Even with a single species and single toxin, the variability in acute

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Table 3. Mean Values of the Water Quality Variables Monitored during the Chronic Sulfate Toxicity to the Zebrafish Experiment

variables	control	250	500	1000	1500	2000	2500	3000	recommended values
temperature (°C)	22.95	23.28	22.85	22.41	23.04	27.12	27.65	28.22	20−30
dissolved oxygen (mg/L)	8.02	8.22	8.26	8.29	7.97	7.52	7.57	7.48	>5
pH	8.59	8.55	8.65	8.63	8.56	8.33	8.35	8.43	6−9
alkalinity (mg $CaCO_3$/L)	203	237	257	260	204	162	152	166	50−450
hardness (mg $CaCO_3$/L)	226	234	220	232	221	235	233	225	50−450
ammonia (mg N/L)	0.45	0.62	0.40	0.75	0.83	0.59	0.62	0.58	<2
nitrite (mg N/L)	BDL[a]	BDL	BDL	BDL	BDL	BDL	BDL	BDL	<0.2
nitrate (mg N/L)	0.52	3.30	2.17	1.86	1.46	1.57	2.09	1.85	<3

[a]BDL: below detection limit.

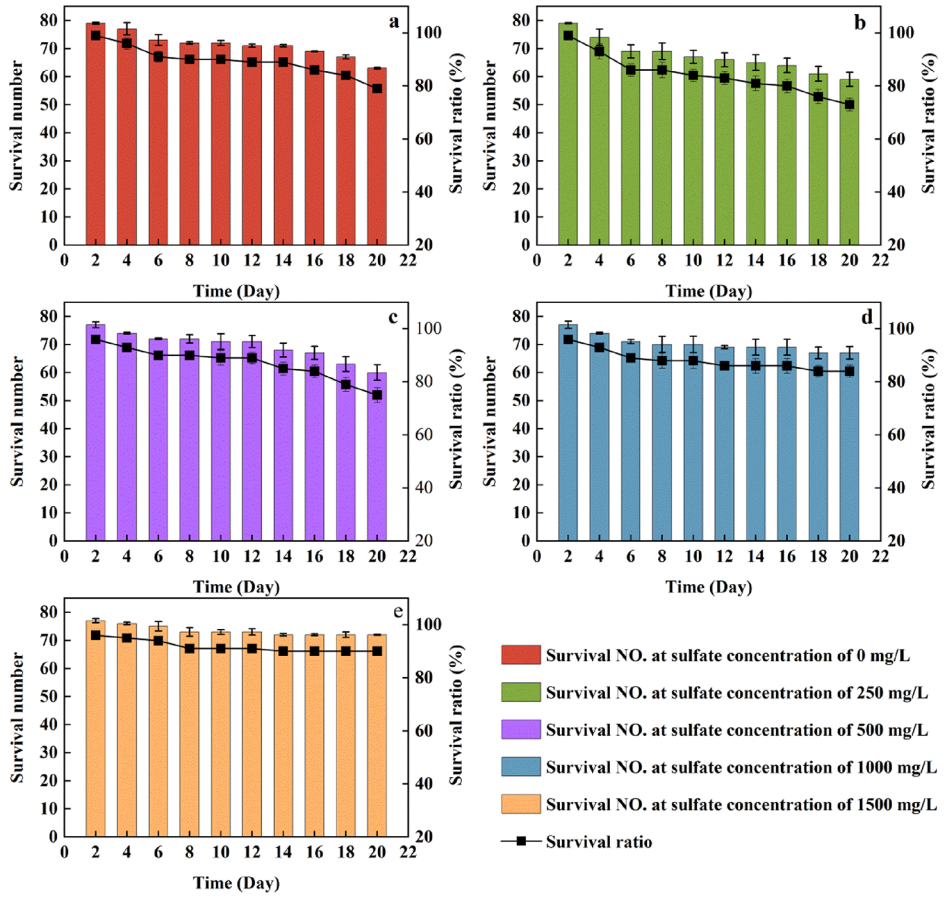

Figure 1. Survival ratio and number of zebrafish at different sulfate concentrations in the first stage: (a) 0 mg/L sulfate; (b) 250 mg/L sulfate; (c) 500 mg/L sulfate; (d) 1000 mg/L sulfate; (e) 1500 mg/L sulfate.

toxicity depends on the size, age, species tested, and other factors.[18] Previous studies have shown that water hardness, chloride content, and acclimation may affect acute toxicity to the fish.[19,20] The acute toxicity of sulfate to the same species of fish was also different in water of similar hardness. Other researchers have reported different results. Wang et al. conducted an acute toxicity study of sulfate on four freshwater organisms and concluded that the 96 h LC_{50} for larval fathead minnow was 4833 mg/L,[4] which is higher than the maximum sulfate concentration of 3200 mg/L used in this study. This might be because the sulfate concentration used in this study was not high enough, so the acute toxicity of sulfate to zebrafish could not be obtained. A higher sulfate concentration should be considered in future studies on the acute toxicity of sulfate to zebrafish.

The mortality of zebrafish increased with time at all concentrations, and the toxicity of sulfate to zebrafish had a significant time effect. The mortality of zebrafish at sulfate concentration of 0−800 mg/L was higher than that at sulfate concentrations of 1600 and 3200 mg/L. Zebrafish as well as *Penaeus monodon* can live in both fresh water and sea water,[21] so their responses to changes in salinity may be similar. In addition, the tolerance of juvenile *P. monodon* to $NaNO_3$ increases with increasing salinity.[22] In this experiment, addition of the Na_2SO_4 stock solution increased the salinity of the solution, so that the survival ratios of the zebrafish at 1600 and 3200 mg/L sulfate concentrations were higher under short-term sulfate exposure.

2.2. Chronic Sulfate Toxicity to Zebrafish. During the two stages of the chronic sulfate toxicity experiment, the water

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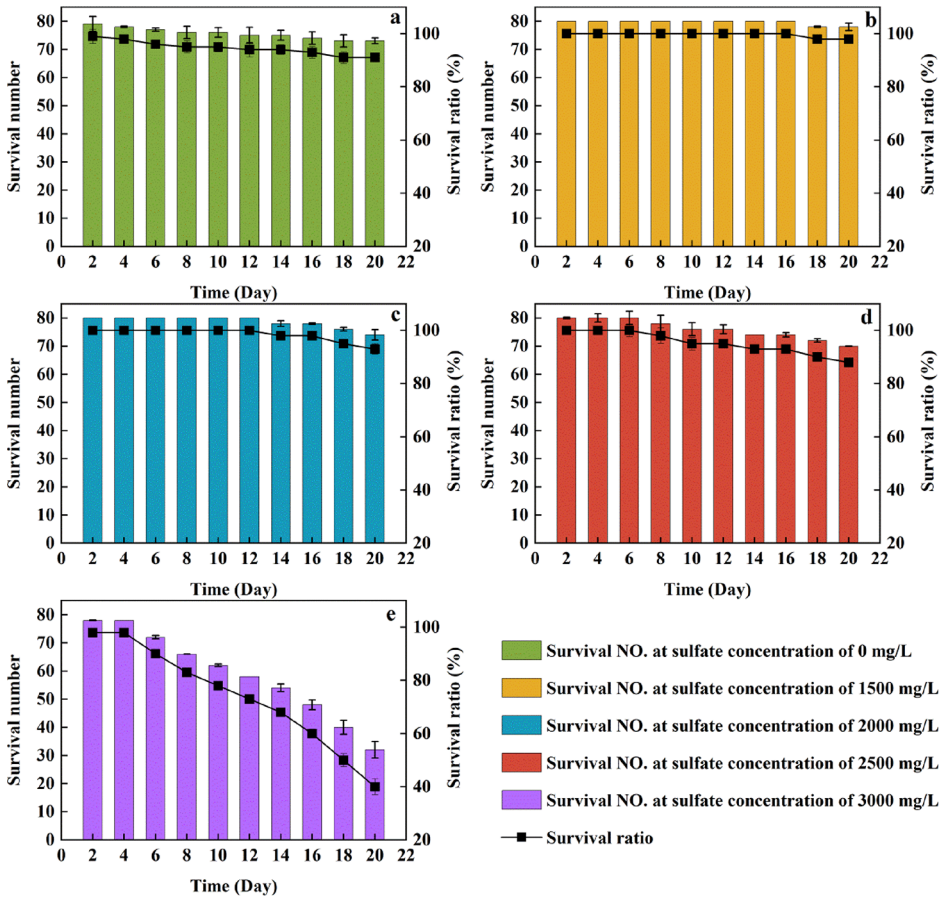

Figure 2. Survival ratio and number of zebrafish at different sulfate concentrations in the second stage: (a) 0 mg/L sulfate; (b) 1500 mg/L sulfate; (c) 2000 mg/L sulfate; (d) 2500 mg/L sulfate; (e) 3000 mg/L sulfate.

quality remained stable within the range suitable for fish survival (Table 3). As in the acute toxicity assay, the change in salinity was also due to addition of the Na_2SO_4 stock solution.

As shown in Figure 1, during the first phase of the chronic toxicity test (21 days), the survival ratio of zebrafish decreases with time. During the following experiment of stage 1, the increase in fish mortality at 0, 250, and 500 mg/L sulfate was more significant than that at 1000 and 1500 mg/L (Figure 1a–c). After the 21 day toxicity test, the survival ratio of the 250 mg/L experimental group was the lowest (74%), and the survival ratios of 500 mg/L experimental groups was 75%. The survival ratios of fish in the 1000 and 1500 mg/L experimental groups were higher (84% and 90%, respectively) (Figure 1d,e). The results show that sulfate significantly affected the survival ratio of the zebrafish (ANOVA, $p < 0.05$) and, except for the control group, when the sulfate concentration ranged from 250 to 1500 mg/L, the survival ratio of the fish increased with the increase in the sulfate concentration. Similar results were found in the acute toxicity test. A reasonable explanation is that zebrafish, like tilapia, is a euryhaline fish and performs better in brackish water.[23]

On the other hand, combined with the toxicity mechanism of salts: (1) osmotic stress, (2) specific ion toxicity,[24] the toxicity of sulfate tended to osmotic stress. Sulfate can interfere with the osmotic pressure of a biological cell and disturbs the water balance of the cell, thus exerting a toxic effect on organisms. Furthermore, the change in sulfate toxicity to fish may be due to the competitive rejection of other ions.[25] In the case of low osmotic pressure, that is, the control group in this study, the water content in gill cells increased, and some ions were lost,[26] which led to the increase of fish mortality. The moderate increase in the salinity of the environment seemed to reduce the osmotic pressure difference between the environment inside and outside the gills of the fish, allowing the fish to grow in a more favorable environment.

Sulfate exposure (1500−3000 mg/L) had a very significant effect on the zebrafish survival ratio (ANOVA, $p < 0.01$). During the first 12 days of the second stage of the experiment, no fish died at sulfate concentrations of 1500 and 2000 mg/L (Figure 2b,c), which indicates that zebrafish might have a high tolerance to sulfate at these concentrations. A rapid decrease in the fish survival ratio was observed at the sulfate concentration of 3000 mg/L. The survival ratio of the fish reached the highest (98%) when the sulfate concentration was 1500 mg/L (Figure 2b). When the sulfate concentrations increased to 2000 and 2500 mg/L, the fish survival ratios dropped to 93% and 88%, respectively (Figure 2c,d), while at 3000 mg/L sulfate, the fish survival ratio was the lowest (40%) (Figure 2e). The survival ratio and number of zebrafish decreased with the increase in sulfate concentration, and the survival ratio of zebrafish decreased with the exposure time (Figure 2). The higher sulfate concentration led to higher zebrafish mortality. At this stage, the survival ratio of the experimental group without sulfate addition (0 mg/L) was lower than that with 1500 and 2000 mg/L sulfate, which demonstrates again that zebrafish are more suitable for living in an aquatic environment of a certain salinity. However, with the further increase in salt of the aquatic environment, the osmotic pressure balance of fish gills

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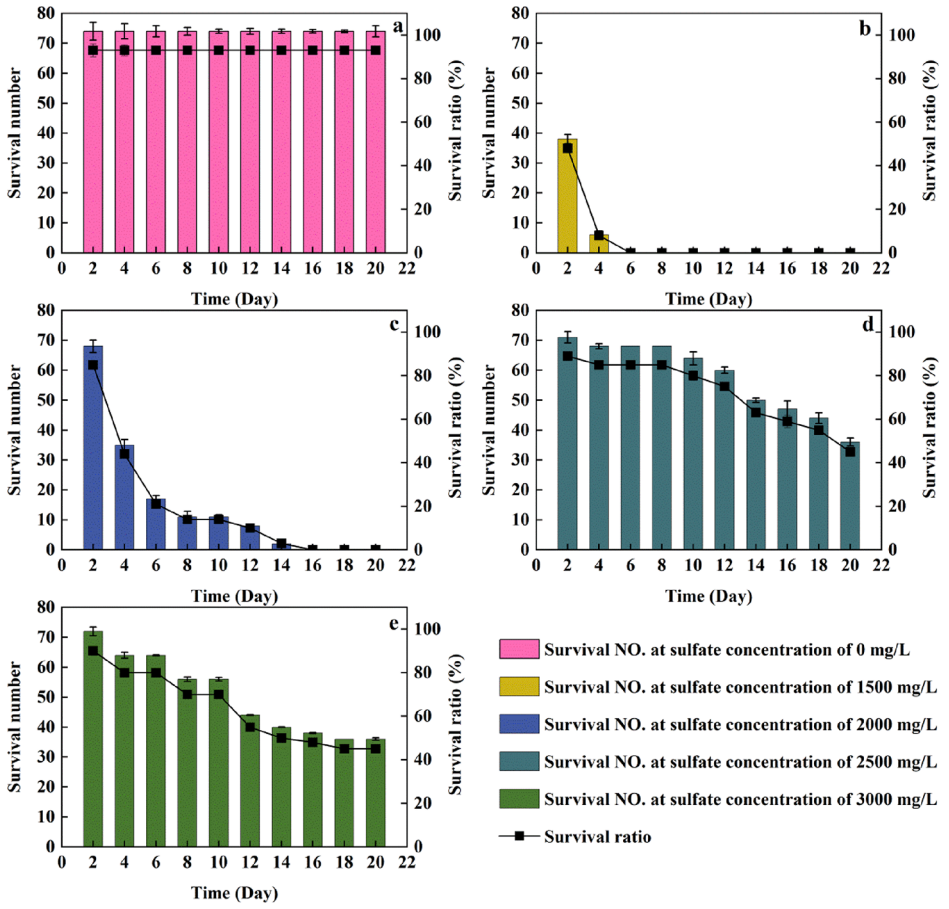

Figure 3. Survival ratio and number of zebrafish at different sulfate concentrations in the ion balance experiment: (a) 0 mg/L sulfate and 0 mg/L KCl; (b) 1500 mg/L sulfate and 2220 mg/L KCl; (c) 2000 mg/L sulfate and 1480 mg/L KCl; (d) 2500 mg/L sulfate and 740 mg/L KCl; (e) 3000 mg/L sulfate and 0 mg/L KCl.

was destroyed. Excessive external osmotic pressure results in water loss due to the difference between the internal and external ambient ion concentrations.[27] Changes in salinity during the experiment also affected the experimental results. Salinity affects water quality, thus affecting the development and physiological conditions of fish.[28]

2.3. Ion Balance Experiment. In order to eliminate the ion imbalance in the fish caused by the increase in Na^+, the relative content of KCl was added during an ion balance experiment. Figure 3 shows the survival ratio and number of living zebrafish at different sulfate concentrations during the experiment. After 21 days of experimentation, the fish survival ratio was up to 93% when the sulfate concentration was 0 mg/L (Figure 3a); all fish were dead in the 1500 and 2000 mg/L sulfate groups; and the survival ratio was 45% for both the 2500 and 3000 mg/L sulfate groups (Figure 3d,e). Results show that a higher fish mortality was observed with more added KCl. This was contradictory to the results from the second phase of chronic toxicity experiments, where fish mortality increased with sulfate concentrations over 1500 mg/L sulfate. One possible reason for this was that K^+ affected the toxicity of sulfate. The higher the concentration of K^+ resulted in stronger the sulfate toxicity to the zebrafish. K^+ is a kind of fixed cation in animals and participates in the metabolism of water and salt, the maintenance of osmotic pressure balance and acid−base balance, and a wide range of physiological and metabolic processes. Therefore, the relative content of K^+ has an important effect on the body metabolism.[29] The imbalance of K^+ affected the activity of fish and even caused death.

The internal and external factors affecting the ion balance, acid−base balance, and osmotic pressure balance in animals are complex. There are many reasons for the differing results, such as the physiological stage of the animals, diseases, water quality, and environmental conditions. In this experiment, the total amount of ions was made consistent to eliminate the effect of ion imbalance, but other factors, such as the major ion composition, were not considered. It has been shown that Na^+ and K^+ are the main ions affecting the toxicity of ammonia to *Hyalella*.[30] In addition to sulfate toxicity, being the main cause of zebrafish death, the ion composition and its relative ratio to different ions, excluding Na^+, K^+, Cl^-, Mg^{2+}, and Ca^{2+}, may also cause the death of aquatic organisms.

2.4. Histopathological Evaluation of the Gill. Except for the control group, zebrafish showed different degrees of abnormal behavior and pathological changes after long-term exposure to sulfate. Zebrafish showed various symptoms, such as slow swimming, sinking to the bottom, large opening and closing range of gills, and side swimming, and with the increasing sulfate concentration, the severity of the symptoms increased and became serious. In addition, the body shape also changed for some living fish. Zebrafish exposed to low sulfate concentrations showed slight spinal curvature and tail injury, whereas those exposed to high sulfate concentrations showed serious spinal curvature, erosion of fins, and redness of the gills. The gills of teleost fish play a key role in organisms. Gills not

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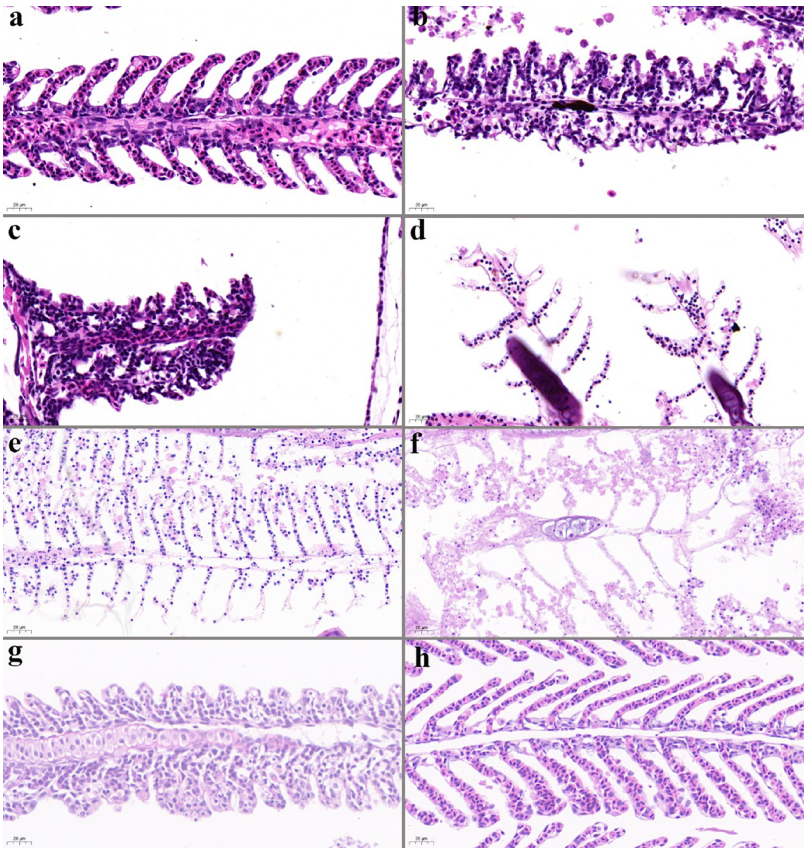

Figure 4. Gills of zebrafish exposed to different concentrations of sulfate: (a) gills in control water; (b) 500 mg/L sulfate; (c) 1500 mg/L sulfate; (d) 3000 mg/L sulfate, fish with marked spinal curvature and fin erosion; (e) 1500 mg/L sulfate and 2220 mg/L KCl; (f) 2000 mg/L sulfate and 1480 mg/L KCl; (g) 2500 mg/L sulfate and 740 mg/L KCl; (h) 3000 mg/L sulfate and 0 mg/L KCl (survival).

only play a key role in gas exchange, removal of nitrogen-containing waste, and acid−base balance, but they also play an important part in ion exchange and osmotic adjustment.[31] Gill epithelial cells, which are directly in contact with the external environment, are very sensitive, and slight damage may destroy their regulatory function, leading to dyspnea.[32] The observed large opening and closing of fish gills may signify that the gill epithelial cells were damaged, which could be confirmed by the tissue section of the gill.

In the control group, there were few or no changes in the gill tissue structure. Long-term exposure to sulfate caused congestion in the zebrafish gills, with small pieces of gills bent and falling off and necrosis of the gill epithelium. In the experimental group with 500 mg/L sulfate, the gills were obviously bent and swollen, some gills fell off, and adjacent gills merged (Figure 4b). Twig bending, congestion, swelling, and shedding were also observed in the 1500 mg/L sulfate experimental group. In addition, pathological changes, such as vascular rupture and epithelial cell proliferation, also appeared (Figure 4c). In the zebrafish exposed to 3000 mg/L sulfate, which also showed spinal curvature, the gill tissue damage was aggravated, including necrosis, hyperplasia, stratum corneum bulge, lamellar fusion, pinocytosis, and hyperplasia in the cartilage center (Figure 4d). Lamellar fusion, gill filament deformity, lamellar curl, and epithelial cell necrosis were observed in zebrafish exposed to 1500 mg/L sulfate and 2220 mg/L KCl (Figure 4e). In the 2000 mg/L sulfate and 1480 mg/L KCl group, in addition to more serious gill filament deformity, lamellar curling, and fused gill tissue damage, hypertrophy and proliferation of the cartilage center cells were also observed (Figure 4f). In the group exposed to 2500 mg/L sulfate and 740 mg/L KCl, significant structural changes were observed in the gill tissue with swollen gill epithelium and seriously proliferated interlayer epithelium (Figure 4g). The gill tissue of zebrafish exposed to 3000 mg/L sulfate and 0 mg/L KCl showed rod-shaped tips and edema and proliferation of gill epithelial cells (Figure 4h).

Damage to the fish gills, which was mainly reflected in two categories, was due to changes in the water environment conditions. First, direct damage occurred to the fish gills, including the necrosis and shedding of gill epithelial cells. The dyspnea identified in the experiment, and the necrosis and shedding of epithelial cells observed in the tissue sections, represented direct damage to the fish gills caused by sulfate. Second, defense reactions also caused damage, including proliferation of gill filament epithelial cells and edema of the respiratory epithelium in the gill piece.[33] For example, when juvenile zebrafish were exposed to nitrate for a long time, gill filaments were damaged by defensive reactions, including oedema, hemorrhages, hyperplasia of epithelial cells, and necrosis.[34] In the presence of silver nanoparticles, the gills of African catfish showed some direct damage and damage caused by defense reactions, such as subepithelial edema, epithelial bulge, interlayer epithelial hyperplasia, epithelial cell necrosis, and secondary lamellar curl.[35] In this study, gill epithelial cell proliferation and edema appeared in the fish gills of all treatments, while more serious injuries, such as lamellar fusion

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and necrosis, were observed with exposure to 3000 mg/L sulfate.

At present, there is little research on the toxicity of sulfate to fish, and the toxicity mechanism of sulfate is still unclear. According to the toxicity of nitrogen, nitrate, and nitrite, sulfate may damage the central nervous system[9] and immune system of fish[36,37] and affect their cardiovascular function and endocrine and excretion processes,[38] thus causing toxic damage to fish. Future research on sulfate toxicity can focus on these aspects.

3. CONCLUSIONS

Zebrafish had a high tolerance to sulfate, with no acute zebrafish toxicity in the range 200−3200 mg/L sulfate. The zebrafish morality decreased with increasing sulfate concentration in the range of 250−1500 mg/L; however, this decreased with the increasing sulfate concentration from 1500 to 3000 mg/L. After long-term exposure to sulfate, zebrafish showed varying degrees of behavioral abnormalities and pathological changes. The behavioral abnormalities included slow swimming; bottom settling; large opening and closing of gills; and lateral swimming. In addition, with an increasing sulfate concentration, this phenomenon was more prevalent and became more serious. In all treatments, gill epithelial cell proliferation and edema were observed, and more severe lesions, such as lamellar fusion and necrosis, were observed at 3000 mg/L sulfate. The results of the ion balance experiment showed that the greater the KCl added, the higher the zebrafish mortality. Current ion balance research has only examined the same amount of total ions and is limited by the possible effect of major ion composition. Thus, further ion balance studies should be carried out to assess the effect of ion composition on aquatic organisms health.

4. MATERIALS AND METHODS

4.1. Fish Acquisition and Acclimatization. Zebrafish (*Danio rerio*) with an average length of 2−3 cm were collected from Shanghai Dashi fishery farm and transported to the laboratory in small aerated polythene bags containing water from the collection site. One month-old zebrafish juveniles were used in this experiment. Before experimentation, the fish were kept in a water tank containing 200 L of dechlorinated water for 2 weeks to acclimatize them to the laboratory conditions. During the acclimatization period, a natural photoperiod was employed and the mean water quality parameter values were determined as follows: temperature 20 ± 0.5 °C, pH 8.5 ± 0.2, dissolved oxygen (DO) >5 mg/L, and alkalinity 250 ± 20 mg/L $CaCO_3$. The fish were fed once per day with commercially available food (crude protein ≥38%, crude fat ≥3.0%, crude fiber ≤8.0%, crude ash ≤15%, moisture ≤10%, calcium ≥1.2%, phosphorus ≥0.4%, lysine ≥1.5%).

4.2. Preparation of Test Water and Test Stock Solution. The test water (control water), which was municipal tap water from the public supply, was aerated for more than 1 week under laboratory conditions to remove chlorine and avoid affecting the survival of fish. The DO, pH, conductivity, hardness, and alkalinity were measured in the test water. The water quality of the experimental water met the survival needs of the zebrafish in terms of temperature (20−30 °C), pH (6.5−8.5), DO >5, alkalinity (250 ± 20 mg/L as $CaCO_3$), and hardness (220 ± 20 mg/L as $CaCO_3$), SO_4^{2-} (25 ± 2 mg/L). A sulfate stock solution of 10,000 mg/L was prepared by adding 147.9167 g of sodium sulfate (Na_2SO_4) to 1 L of deionized water. Since sodium ions (Na^+) are less toxic to aquatic organism than the other cations,[19] Na_2SO_4 was selected to prepare the stock solution. The experimental solution was obtained by adding a corresponding volume of sulfate stock solution to test water.

4.3. Experimental Design. The experimental protocol was approved by the Animal Care and Use Committee of Henan University of Technology.

4.3.1. Acute Toxicity. An acute toxicity test was performed to reveal the effects of various sulfate levels on zebrafish. The tested sulfate concentrations were 0, 200, 400, 800, 1600, and 3200 mg/L, and the test duration was 96 h. At the beginning of the acute toxicity tests, 20 zebrafish were impartially transferred to each of 3 replicate 1000 mL glass beakers containing 600 mL of test solution. The zebrafish were not fed during the acute toxicity experiment. During the experiment, water quality parameters, specifically temperature, pH, DO, and alkalinity, were measured daily at 10:00 am and 7:00 pm. To exclude the toxicity of nitrate and ammonia nitrogen on fish growth, nitrate and ammonia nitrogen concentrations were measured at the beginning and end of the experiment. The number of immobilized and dead fish was recorded daily, and then the dead fish were removed. The fish mortality was quantified at 24, 48, 72, and 96 h.

4.3.2. Chronic Exposure. Sulfate concentrations in aquatic environments are usually low (below 580 mg/L), but natural sulfate levels are in excess of 3000 mg/L in some lakes or seawater.[1] Therefore, the initial concentration for the experiment was set as 250 mg/L, and the maximum concentration of 3000 mg/L was set to study the toxic effects of sulfate on aquatic organisms. Furthermore, the experimental group without sulfate addition was also used as the control. The purpose of phase 1 was to reveal the effect of low sulfate levels (0, 250, 500, 1000, and 1500 mg/L) on fish growth performance. The purpose of phase 2 was to determine the effects of high sulfate concentration (0, 1500, 2000, 2500, and 3000 mg/L) on the aquatic organism. Each treatment was performed in duplicate, and each phase lasted 21 days. Zebrafish (80 individuals) were exposed to the designed sulfate-polluted water in aerated 25 L glass tanks ($L \times W \times H$: 42 × 23 × 26 cm) for phase 1 and phase 2. In addition, each fish tank was equipped with an automatic feeder and a filter for removing uneaten food and fish feces. Every 12 h, the fish were fed 2 g of food (about 1%−2% of their body weight). In order to maintain the required concentration of test compounds in the aquaria, 50% of the aquarium water was replaced every 10 days with water containing the required compound concentrations. Water parameters, including temperature, DO, conductivity, and pH, were recorded daily at 2:00 pm. Growth performances, specifically mortality, swimming abnormalities, and lesions, were observed and recorded for each phase of the experiment. During the daily observation, the dead fish were recorded and then removed and immediately placed in 4% formalin to assess the body tissues.

4.3.3. Ion Balance Experiment. In this study, Na_2SO_4 was dissolved in deionized water to prepare a stock solution. The stock solution was diluted in experimental water to obtain the corresponding sulfate concentration, which increased the content of Na^+, thereby increasing the ratio of Na^+/K^+. Studies have shown that an increase in the Na^+/K^+ ratio leads to an increase in the osmotic pressure adjustment pressure of aquatic organisms and subsequently death.[39] Therefore,

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potassium chloride (KCl) was added to balance the total ions. In this study, the ion balance experiment was carried out using the high sulfate concentration treatments of 0, 1500, 2000, 2500, and 3000 mg/L. Concentrations of KCl added were 0, 2220, 1480, 740, and 0 mg/L. The specific experimental scheme is shown in Table 4. The experimental conditions were the same as those of the chronic toxicity experiment.

Table 4. Ion Balance Experimental Design

concentration of sulfate (mg/L)	control	1500	2000	2500	3000
concentration of $NaSO_4$ added (mg/L)	0	2219	2959	3699	4439
concentration of KCl added (mg/L)	0	2220	1480	740	0
total salinity (mg/L)	0	4439			

4.4. Histopathological Analysis. In order to further study the effect of sulfate on fish tissues at various concentrations, fish gills were histologically analyzed. The dead fish were collected from the corresponding tanks, and the gill was removed for histopathological analysis. The gill samples were immediately placed in 4% formalin, fixed for more than 24 h, and then transferred to 70% ethanol. Afterward, the tissues were dehydrated in a continuous ethanol gradient of 75%, 85%, 90%, 95%, and 100% and then cleared in xylene, infiltrated with paraffin, and embedded in paraffin blocks. The blocks were placed in a 20 °C freezing table, and then the tissue chip wax blocks were sliced with a paraffin slicer (Leica, HistoCore AUTOCUT) to a thickness of 4 μm. Subsequently, the tissues were merged for staining in hematoxylin solution for 3−5 min. The prepared tissues were observed with a microscope (Nikon Eclipse E100).

4.5. Chemical Analyses. Water samples were collected from each tank with a syringe for water quality analysis, including temperature, DO, conductivity, pH, alkalinity, hardness, NO_2^--N, NO_3^--N, NH_4^+-N, and SO_4^{2-}. During long-term exposure, the conductivity was measured regularly to monitor the ion concentration. The samples were filtered through a 0.45 μm membrane filter before detection. DO, pH, and conductivity were measured daily using a handheld multiparameter water quality analyzer equipped with the Thermo Scientific Eutech DO 6+, pH 6+, and COND 6+ meters, respectively. Alkalinity and hardness were measured by standard methods (acid base indicator titration; EDTA complexometric titration). NO_2^--N, NO_3^--N, and NH_4^+-N were measured by ultraviolet spectrophotometry (UV-5900PC, METASH, Shanghai). SO_4^{2-} was determined by ion chromatography (ICS-900, Thermo, US).

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Notes

The authors declare no competing financial interest.

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