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Assessment of Acute Toxicity, Behaviours and Water Parameter Correlations in Nile Tilapia (*Oreochromis niloticus*) Exposed to Iron Salts

(Penilaian Ketoksikan Akut, Tingkah Laku dan Korelasi Parameter Air pada Tilapia Nil (*Oreochromis niloticus*) Terdedah kepada Garam Besi)

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ABSTRACT

The study determined the acute toxicity levels and behaviors of Nile tilapia after 96 h exposure to iron salts. It determined the significant correlations (Pearson's r) between the water parameters, behaviors, and toxicity levels at varied time intervals and concentrations. This study adopted an experimental design framework, randomized triplicate sampling, and OECD Test Guideline No. 203. The acute toxicity test was done in a static method, behavioral clinical signs and opercular beats. Water parameters of pH, temperature, dissolved oxygen (DO), and total organic carbon (TOC) were measured using standard procedures of the OECD Test Guideline No. 203 and Association of Official Analytical Chemists (AOAC). The findings showed the lethal concentration (LC_{50}) and lethal time (LT_{50}) of total ferrous and ferric sulfate were 135.6 mg/L, 9.9 mg/L, and 340 h and 216 h, respectively, through probit analysis. Behaviors were abnormal bottom distribution, hypoventilation, abnormal pigmentation, and bioaccumulation of ferric ions. Opercular beats (OB) decreased as the concentration increased after 96-h exposure. Strong correlations ($\alpha = 0.05$) were observed between concentration and the water parameters OB and pH in the ferric sulfate solutions, between pH, hardness, TOC, OB, and DO in ferrous sulfate solutions. The findings showed that ferric sulfate poses a greater risk than ferrous sulfate to test animals, implying its use and discharge should be limited in aquatic environments. The water parameters that significantly affected the toxicity levels must be monitored and regulated to mitigate potential adverse effects on aquatic ecosystems.

Keywords: Acute toxicity; behaviours; iron salts; water parameters

ABSTRAK

Kajian ini menentukan tahap ketoksikan akut dan tingkah laku ikan tilapia Nil selepas 96 jam terdedah kepada garam besi. Ia menentukan korelasi yang ketara (Pearson's r) antara parameter air, tingkah laku dan tahap ketoksikan pada selang masa dan kepekatan yang berbeza. Kajian ini menggunakan rangka kerja reka bentuk uji kaji, pensampelan tiga kali ganda rawak dan Garis Panduan Ujian OECD No. 203. Ujian ketoksikan akut dilakukan dalam kaedah statik, tanda klinikal tingkah laku dan denyutan operkular. Parameter air pH, suhu, oksigen terlarut (DO), dan jumlah karbon organik (TOC) diukur menggunakan prosedur standard Garis Panduan Ujian OECD No. 203 dan Persatuan Ahli Kimia Analitik Rasmi (AOAC). Hasil menunjukkan kepekatan maut (LC_{50}) dan masa maut (LT_{50}) bagi jumlah ferus dan ferik sulfat masing-masing ialah 135.6 mg/L, 9.9 mg/L dan 340 jam dan 216 jam melalui analisis probit. Tingkah laku adalah taburan bawah yang tidak normal, hipoventilasi, pigmentasi yang tidak normal dan biotumpukan ion ferik. Denyut operkular (OB) menurun apabila kepekatan meningkat selepas pendedahan selama 96 jam. Korelasi yang kuat ($\alpha = 0.05$) diperhatikan antara kepekatan dan parameter air OB dan pH dalam larutan ferik sulfat, antara pH, kekerasan, TOC, OB dan DO dalam larutan ferus sulfat. Hasil menunjukkan bahawa ferik sulfat menimbulkan risiko yang lebih besar daripada ferus sulfat untuk menguji haiwan, membayangkan penggunaan dan pelepasannya harus dihadkan dalam persekitaran akuatik. Parameter air yang mempengaruhi tahap ketoksikan dengan ketara mesti dipantau dan dikawal untuk mengurangkan potensi kesan buruk ke atas ekosistem akuatik.

Kata kunci: Garam besi; ketoksikan akut; parameter air; tingkah laku

INTRODUCTION

Iron waste from cutleries from wrought iron, steel and other alloys containing iron are in its elemental form when released in the environment. The oxidation state of Fe (II) or Fe (III) depends on the redox condition of the environment. Generally, in anaerobic conditions like in waterlogged soils, Fe (II) forms, and aerobic condition like in fishponds, Fe (III) forms (Luther 2016). Water in contact with air is oxidized and precipitation of ferric hydroxide occurs naturally (Hem & Skougstad 1960). Ferric hydroxide precipitates can bind physically in gills that can clog and inhibit respiration in fish (Slaninova, Machova & Svobodova 2014). Fish caught from a fishpond near a blacksmith shop had iron deposits in its gills and tail.

Tabaco City in Albay, Philippines, is known for its cutlery industry. The name Tabaco was coined after 'Tabak' or bolo, a metal produced by skilled blacksmiths (Besmonte 2020). There are 137 registered blacksmiths in 27 blacksmith shops at various barangays in the city (CBMS 2021). This industry generates iron as a waste product. These include three forms of iron that exist in nature: iron (II), iron (III), and iron (IV). Reduced iron (II) when exposed to O_2 and H_2O transform into iron (III) (Chang 2015) or insoluble hydroxide and oxyhydroxide in an acidic aqueous environment (Hem 1985; Kimball, Walton-Day & Runkel 2007). The reduced iron (II) can also be oxidized by iron bacteria into insoluble ferric oxide [iron (IV)] in an aqueous environment with low pH and low temperature (Baker, Martin & Davies 1997; Lappivaara, Kiviniemi & Oikari 1999). Iron fillings accumulate in soil and bodies of water. Studies conducted in the United Kingdom and Australia reported a large amount of iron near cutlery that exceeded the allowable limit (Harber & Froth 2001; Strezov & Chaudhary 2017). Bolo making in Tabaco City existed during the pre-Spanish era, as accounted in the folk story 'Osipon', where the chieftain of the place shouted 'Tabak ko, Tabak ko' when the Spanish conquistadores stood dangerously close to a young woman who was the daughter of the chieftain. There is no study conducted on the effects of iron deposits from blacksmiths shops in Tabaco City to fish health.

Iron is essential for life; however, it can cause oxidative stress and disruption of sodium balance when present in large amounts (Gonzalez, Grippo & Dunson 1990; Lasocki, Gallard & Rineau 2014). The proximity of blacksmiths shops to fishponds caught the attention of the author as more fishponds are built based on information from the Tabaco City Agricultural Office. In fishponds, dissolved iron is less than the normal amount (5 μg/L). However, when the fishpond is near a blacksmith shop, the amount of iron can be more than the accepted amount. The presence of iron deposits in the gills of Nile tilapia prompted the author to study the effects of ferric and ferrous salts to Nile tilapia. The accumulation of ferric and ferrous salts in bodies of water was simulated in an experimental set-up to determine its toxicity to Nile tilapia which is the most requested species at the Bicol University Tabaco hatchery.

An acute toxicity test evaluates the concentration to kill 50% of the fish (LC_{50}) when exposed to a test chemical for 96 h under static or flow-through conditions. Lethal time 50 (LT_{50}) is the time required to kill half of the population of the test animals (OECD 2019). The conditions during the test simulate the pollutants existing in the natural environment to assess their impact on aquatic organisms and water parameters (Qu et al. 2013). The primary objective of the study was to determine the acute toxicity levels of ferric and ferrous sulfate and the water parameters during the acute toxicity test. The behavior of fish during the acute toxicity test predicts moribundity and death as experimental endpoints as mentioned in the OECD Test Guideline No. 203 (2019). The significant correlations of water parameters were evaluated to predict the effect of water parameters on acute and behavioral toxicity of the test chemicals. There was no previous study conducted on the toxicity of iron to Nile tilapia in the study setting and the determination of water parameters as affected by iron salts. Identifying the LC_{50} and LT_{50} of the iron salts will provide information on the capacity of Nile tilapia to survive in fishponds near blacksmiths shops. The behavior and water parameters that were identified to affect the toxicity can be monitored in the fishponds to prevent the contamination of the iron salts to fish and its consumers.

CONCEPTUAL FRAMEWORK

The core concept of the study involves the acute toxicity levels (LC_{50} and LT_{50}) of the toxicants (ferric and ferrous sulfate) to Nile tilapia within 96 h and behavior (clinical signs and opercular beats) and water parameters (pH, temperature, dissolved oxygen, and total organic carbon) that affected the toxicity of the iron salts in aquatic environments. The independent variables were the concentration of ferric and ferrous sulfate in ppm, and the dependent variables were the LT_{50} and LC_{50} and correlations between the acute toxicity levels and water parameters. The mediating variables were the behaviors (clinical signs and opercular beats) and water parameters (pH, temperature, DO, and TOC). Figure 1 describes the relationships between the concentrations of iron salts, water parameters, behaviors, and acute toxicity levels. This framework guides the evaluation of the effects of the iron salts and regulatory measures through behaviors and water parameters to protect aquatic environments. The mediating variables can guide fishpond owners and consumers on the possibility of high iron deposits in the fish pond water and soil that can lead to toxicity.

MATERIALS AND METHODS

ACUTE TOXICITY TEST

The acute toxicity test was conducted at the Wet Laboratory of Bicol University Tabaco, Tabaco City. One hundred twenty (120) test animals of Nile tilapia (*Oreochromis niloticus*) with an average weight of 2.3±0.24 g was used. The species were weighed digitally with a sensitivity of 0.1 mg using an analytical balance (EX324/AD, Ohaus). The average length was 54±2.2 mm as measured by digital calipers (QS-50, Jakemy, China). Limit test was conducted first for 96 h at 100 mg/L to demonstrate the LC_{50} is greater or lesser than the concentration. The

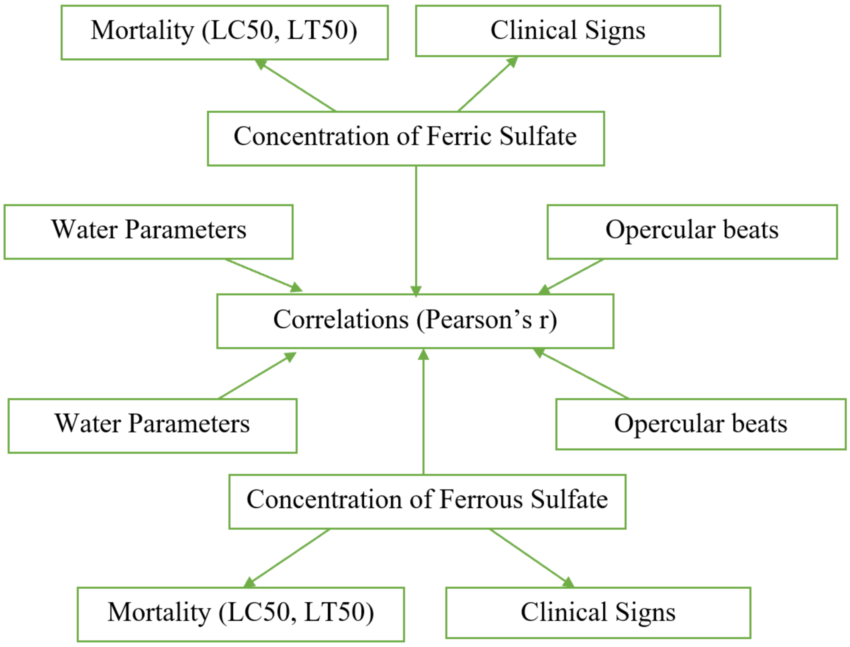

FIGURE 1. Conceptual framework

concentration of the iron salts was adopted from the study of Gemaque et al. (2019). The fish were purchased from the Bicol University Tabaco hatchery, acclimated for seven days and fed at 8:00 and 17:00 daily. The feed was Fry Master Booster 1 with 48% crude protein, 10% crude fat, 5% crude fiber, 16% ash, and 12% moisture.

Before the acute toxicity tests, feeding was stopped for 24 h as recommended by OECD Test Guideline No. 203: Fish Acute Toxicity Testing (2019). The test chemicals were purchased online, with a purity of 99%, and were marked as analytical grade by Spectrum Chemicals. The test solutions were prepared using water treated with AquaSafe (water chlorine remover). The treatments were conducted in glass jars with 6 L aerated capacity for 24 h (ACO-9820 Super Silent Power Air Pump, Hailea). The tilapia fingerlings were divided into 11 groups and added to the ferric and ferrous sulfate solutions, whose concentrations were measured at 0 and 96 h. The utilization of ferric sulfate and ferrous sulfate was due to the location of the study. Tabaco City is on the slope of Mayon Volcano, an active volcano. Active volcanoes emit 6.8 Tg of Sulfur dioxide per year when calm and 11.9 Tg of Sulfur dioxide when erupting (Stoiber, Williams & Huebert 1987). Sulfur dioxide, when oxidized, is converted to sulfur trioxide, and sulfur trioxide in the presence of water is converted to sulfuric acid. In the presence of elemental iron, ferrous sulfate and ferric sulfate will be produced.

Each group had three replicates. Group 1 was the control group (0 mg/L); Group 2 - 1 mg/L ferric sulfate aqueous solution; Group 3 - 3 mg/L ferric sulfate aqueous solution; Group 4 - 7.5 mg/L ferric sulfate aqueous solution; Group 5 - 15 mg/L ferric sulfate aqueous solution; Group 6 - 30 mg/L ferric sulfate solution; Group 7 - 1 mg/L ferrous sulfate aqueous solution, Group 8 - 3 mg/L ferrous sulfate aqueous solution; Group 9 - 7.5 mg/L ferrous sulfate aqueous solution; Group 10 - 15 mg/L ferrous sulfate aqueous solution; and Group 11 - 30 mg/L ferrous sulfate aqueous solution. Ten mL of ammonium acetate buffer was added for each replicate to prevent the sudden decrease in the pH. For freshwater fish like tilapia, a maximum loading of 0.8 g wet weight fish/L for static testing is recommended (OECD 203, 2019). Each aquarium had four (4) test animals and had three (3) replicates per concentration.

BEHAVIOR TESTS

The OECD Test Guideline No. 203 (Table 1) was utilized in observing the clinical signs manifested by the fish throughout the 96-h acute toxicity test. The analysis of opercular beats (OB) of Nile tilapia exposed to ferrous and ferric sulfate was adapted from the study of Edwin et al. (2018). The study of Edwin et al. (2018) utilized the application CapCut that can play videos in slow motion while enlarging the image for a clearer view. The OB of fish was affected when subjected to low temperatures, therefore, analysis of OB will show respiratory stress or metabolic response to toxicants (Tantarpale et al. 2012). After 96 h the surviving tilapia fingerlings from each concentration were kept individually in beaker glass and acclimatized for 10 min before recording the number of beats per minute. The OB was recorded manually with a camera. The recorded videos were imported and slowed into CapCut movie editor for accurate counting. Figure 5 shows the processes involved in analysis of the OB.

TABLE 1. Acute toxicity tests results of test animals (size 14)

Parameter	Ferric sulfate	Ferrous sulfate
LC_0	1.0 mg/L	3.0 mg/L
LC_{100}	17.7 mg/L	43,372.4 mg/L
LC_{50}	9.9 mg/L	135.6 mg/L
LT_{50}	216 h	340 h

WATER PARAMETER TESTS

The pH and temperature were measured by CyberScan pH 11 (Eutech Instruments), dissolved oxygen (DO) by the Winkler method as mLO_2/L by Carpenter (1965), and the water hardness is the total hardness of Ca^{2+} and Mg^{2+} ions in water by complexometric titration (Sumalapao et al. 2017). Light intensity was measured by the application Lux Light Meter (Microstudio34, Google Play Lux). The total organic carbon (TOC) was determined by the colorimetric method of Greenberg (1985), and absorbances of test solutions were measured using the same UV-vis spectrophotometer model in the test solutions' iron content determination. TOC can detect the presence of organic matter that can form complexes in iron that will affect the solubility and movement of the iron complex in water.

TOTAL IRON CONTENT AT 0 AND 96 H

OECD Test Guideline No. 203 mentioned the need to measure the concentrations at the start and end of exposure period for static tests. The total iron content of ferric sulfate and ferrous sulfate aqueous solutions was measured in UV-2505 (Labomed Inc.) following the phenanthroline method of Flinn Scientific (1994).

CORRELATION MATRIXES AND LINEAR REGRESSION

Correlation matrixes were gathered on the data collected at every time interval to determine the relationship of the dependent variable of concentration in ppm, the behaviors and water parameters. The significantly related variables were tested by linear regression for its R, F, and p-value. The statistical analyses were done using IBM SPSS Statistics 20 and Jamovi 2.4.11 at a significance level 0.05 or calculating a 95% confidence interval.

RESULTS AND DISCUSSION

ACUTE TOXICITY TEST

The LC_0 was observed after the 96-h acute toxicity test of Nile tilapia to the various concentrations. It was observed that there was no mortality for the control (0 mg/L) and 1 mg/L after 96 h of exposure of the Nile tilapia to ferric sulfate. The LC_0 for ferrous sulfate was observed after 96 h when there was no mortality for the control (0 mg/L), 1 mg/L, and 3 mg/L (Table 1). The LC_{50} was computed through probit analysis using IBM SPSS Statistics 20. Probit analysis can handle binary data like mortality rates and LC_{50}/LT_{50} due to its binomial response, sigmoid-dose response transition, and capacity to estimate LC_{50}/LT_{50}. With the computed values of LC_{50} of 9.9 mg/L for ferric sulfate and 135.0 mg/L for ferrous sulfate, it can be observed that ferric sulfate is more toxic than ferrous sulfate; it was observed during the acute toxicity test that ferric sulfate formed insoluble precipitates in the gills of the fish. The LT_{50} was computed through probit analysis using IBM SPSS Statistics 20. The LC_{50} of ferric sulfate can kill half of the fish population after 216 h while 340 h for ferrous sulfate. Therefore, ferric sulfate is more toxic to Nile tilapia fingerlings than ferrous sulfate. Short-term exposure to ferric sulfate can lead to significant mortality. Ferrous sulfate requires higher concentrations and longer exposure for similar effects.

BEHAVIOR TESTS

ABNORMAL BOTTOM DISTRIBUTION

After the 96-h exposure, abnormal bottom distribution of the Nile tilapia fingerlings exposed to both toxicants have been observed. Similar abnormalities were also observed in *Tilapia* spp. and *Tilapia zilli* when exposed to iron and other heavy metals (Kusemiju et al. 2022; Mendoza et al. 2023). These observed abnormalities could also be attributed to the effects of heavy metals (Cu, Hg, Pb, and Cd) on Nile tilapia. Specifically, abnormal swimming behaviors may be linked to extensive hepatocyte necrosis observed in the liver tissues of tilapia (Abdel-Hakim et al. 2016). Figure 2 shows the abnormal bottom distribution.

HYPOVENTILATION

Hypoventilation of Nile tilapia fingerlings was also observed, particularly during analysis of opercular beats. Higher concentrations of ferric and ferrous ions were found to induce lower opercular beats per minute. The inhibition of respiratory enzymes, likely due to low pH levels of water with ferric and ferrous iron, may be the main reason for these abnormalities. Inhibition of respiratory enzymes can decrease respiratory movements as suggested by Muthukumar, Anbalagan and Krishnan (2009). The study of Grobler-van Heerden, van Vuren and Du Preez (1991) mentioned the decrease in respiratory movement is one way of acclimatizing of Nile tilapia to iron salts. Figure 3 shows the hypoventilation observed.

ABNORMAL PIGMENTATION (YELLOW DISCOLORATION)

After 96 h of exposure to ferrous and ferric ions, yellow discoloration was observed on Nile tilapia fingerlings, this was also noted in the effects of zero-valent iron nanoparticles (nZVI) (Oladosu, Ayinla & Ajiboye 1994). Studies have shown that nZVI can induce such coloration on the skin of *Tilapia mossambicus*, which may be attributed to oxidative stress. However, based on the water quality parameters, it indicates that dissolved oxygen levels remained within acceptable ranges for *Oreochromis niloticus*. The low dissolved oxygen uptake, leading to yellow discoloration, is likely a result of disrupted gill function due to insoluble ferric and ferrous iron binding to the gills of the fish. Figure 4 shows the abnormal pigmentation or yellow discoloration of the fish.

BIOACCUMULATION OF FERRIC IONS IN FISH

Bioaccumulation of ferric ions in fish is shown in Figure 5. There is evidence of ferric precipitates that bind the gills of Nile tilapia fingerlings after exposure. As indicated by Slaninova, Machova and Svobodova (2014), ferric ions can bind to alkaline gill surfaces of fish. This bioaccumulation process may contribute to observed decrease in ferric concentration and disrupted gill function. The effect of iron bioaccumulation is on the physical blockage and oxidative damage. Iron (III) in ferric hydroxide is insoluble in water according to the solubility rule; hydroxides form precipitates in aqueous solutions except when combined with alkali metal cations, ammonium, calcium, and strontium cations. Iron can oxidize at a lower pH in environmental water in the presence of reactive oxygen species (ROS) that can cause cellular damage (Luther 2016).

OPERCULAR BEATS

The detrimental effects of toxicants are directly linked to opercular beats. Increase in exposure have different effects. Edwin et al. (2018) observed increasing OB in one moth old tilapia in tannery wastewater after thirty (30) days. Whereas, decrease OB was observed by Gabriel and Erondu (2010) when *Clarias gariepinus* were exposed to glyphosate products for ninety-six (96) h. The same observation was made by Gabriel and Okey (2009) when hybrid catfish were exposed to aqueous leaf extracts of *Lepidagathis alopecuriodes*. Therefore, the effect on the OB of fish is dependent on the test chemical and test animal species. In the study, fish exposed to ferric and ferrous toxicants exhibited decreasing opercular beats as the concentration increased after 96-h exposure. This outcome suggests a significant inhibitory effect of the toxicant on the respiratory movements of the fish, as reported by Muthukumar, Anbalagan and Krishnan (2009). This inhibition of enzymes ultimately leads to a decrease in the metabolic activity associated with respiratory movement, as discussed in the same study by Muthukumar, Anbalagan and Krishnan (2009). The ability of tilapia to acclimatize to iron and regulate the concentration after seventy-two (72) h exposure was observed by Grobler-van Heerden, van Vuren and Du Preez (1991). The decrease in OB is one-way of acclimatizing to adapt to the ferric and ferrous salts after 96-h exposure.

WATER PARAMETER TESTS

Tables 2 and 3 show the average measurements of the water parameters measured at 0, 24, 48, 72, and 96 h. For ferric sulfate, there was a direct relationship between the DO level, water hardness, TOC, and concentration of the test chemical. However, there is an inverse relationship between the pH level and ferric sulfate concentrations. The temperature and light intensity were not tabulated because they were not affected by the changes in the concentrations. The total iron content was measured at 0 h and 96 h to observe the changes in the parameters before and after the acute toxicity test.

Table 3 shows the inverse relationship between concentration and DO levels. This indicates reduced oxygen availability for the fish. However, the DO was not affected by increasing concentration of ferric sulfate. As the ferric sulfate concentration rises, the pH decreases making the solution more acidic. Water hardness (measured in ppm) and TOC levels remains relatively stable throughout the 96-h acute toxicity test. Ferric sulfate exhibits acute toxicity to Nile tilapia fingerlings, at higher concentrations the gills were affected as shown in Figure 5. Ferric is the insoluble form of iron that causes oxidative stress and physical damage to gills (Egnew et al. 2021). Ferric oxide is less toxic but can smother eggs or clog gills, affecting gas exchange (Slaninova, Machova & Svobodova 2014). This result was not observed in the ferrous sulfate solution.

IRON CONTENT BEFORE AND AFTER ACUTE TOXICITY TEST

Table 4 shows the change in the total iron content at 0-h and 96-h for all concentrations of ferric sulfate except for the control. It was evident that the concentration of ferric sulfate decreased after the 96-h acute toxicity test. The ferrous sulfate concentrations did not change after the 96-h toxicity test and was not tabulated.

Ferric sulfate ions (Fe^{3+}) can form complexes with sulfate ions (SO_4^{2-}) in most natural and man-made extremely acidic environments. This complexation influences the redox properties of ferric iron. The standard redox couples of both the $Fe(SO_4)_2^-$ and $Fe(SO_4)^+/Fe^{2+}$ couples are more electronegative than that of the uncomplexed metal (Johnson, Kanao & Hedrich 2012). Ferric sulfate will precipitate in the absence of alkalinity in an unpolluted environment (Clarke et al. 1997).

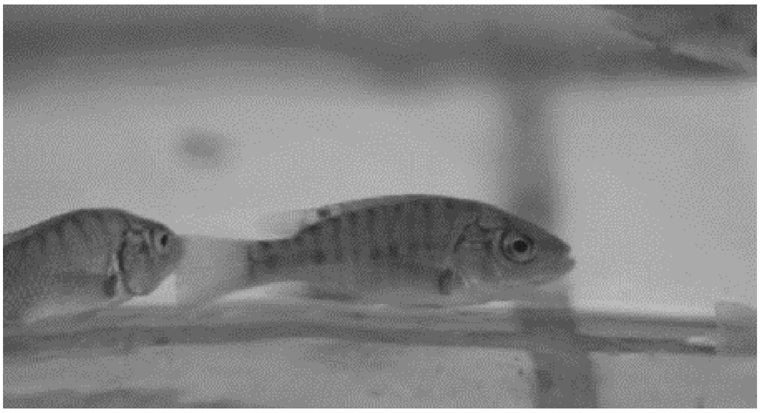

FIGURE 2. Abnormal bottom distribution

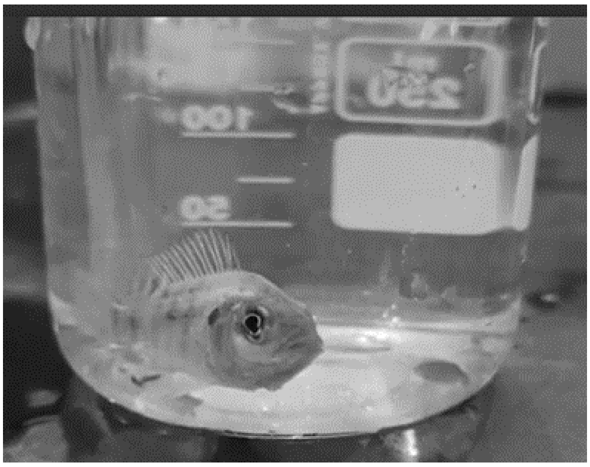

FIGURE 3. Hypoventilation

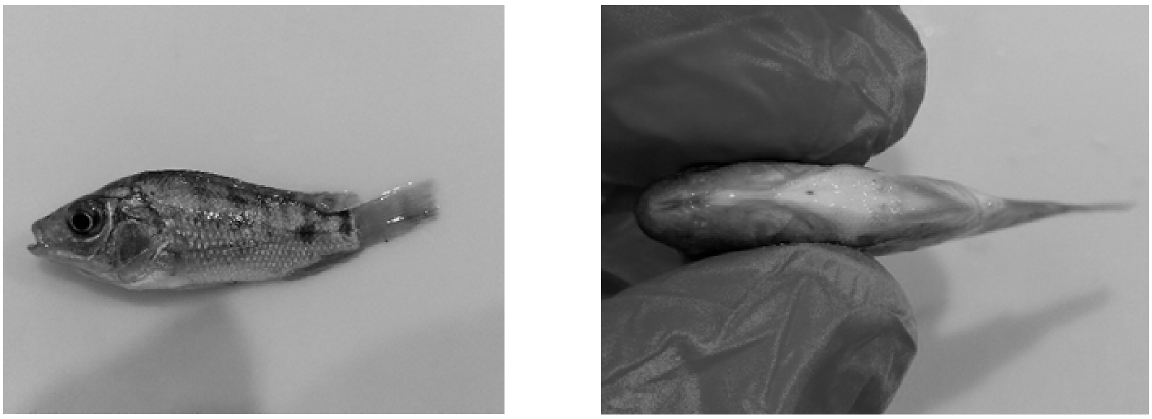

FIGURE 4. Abnormal discoloration

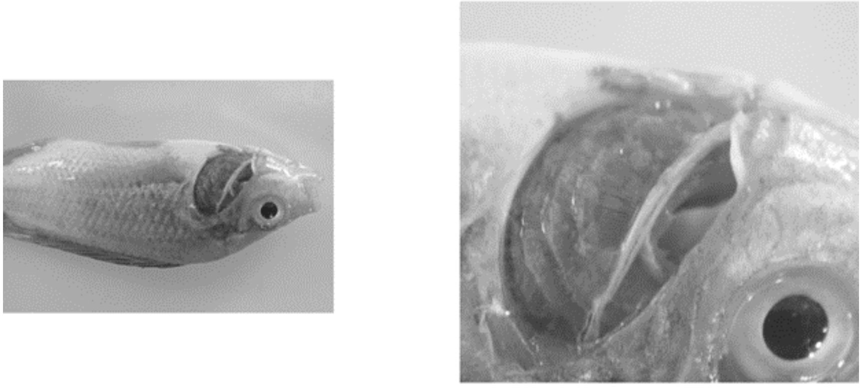

FIGURE 5. Gill filaments covered with insoluble ferric

TABLE 2. Water quality of the varied concentrations of ferric sulfate

Concentration (ppm)	Dissolved oxygen (ppm)	pH	Water hardness (ppm)	Total organic carbon (ppm)
0	8.30±0.274	7.57±0.480	24±9.729	0.478±0.018
1	8.27±2.230	6.26±0.746	25±11.143	0.483±0.007
3	8.43±0.328	5.02±0.920	27±12.639	0.479±0.009
7.5	8.47±0.375	3.62±1.782	40±4.504	0.482±0.004
15	8.63±0.375	3.08±0.141	122±1.020	0.487±0.010
30	8.70±0.325	2.67±0.275	270±7.458	0.493±0.005

TABLE 3. Water quality of the varied concentrations of ferrous sulfate

Concentration (ppm)	Dissolved oxygen (ppm)	pH	Water hardness (ppm)	Total organic carbon (ppm)
0	8.30±0.293	7.57±1.930	24±112.963	0.478±0.005
1	8.07±0.544	7.14±1.793	145±89.581	0.494±0.005
3	7.80±0.530	7.19±0.974	97±31.982	0.490±0.004
7.5	7.70±0.590	7.23±1.657	97±27.968	0.490±0.005
15	7.83±0.598	7.20±2.287	95±14.720	0.489±0.004
30	7.83±0.623	7.39±2.400	98±91.404	0.489±0.005

TABLE 4. Total iron content before and after the acute toxicity test

Concentration (ppm)	0 (h)	96 (h)
0	0	0
1	1.00±0.001	0.99±0.002
3	3.00±0.002	2.99±0.005
7.5	7.5±0.000	7.40±0.003
15	15.0±0.001	14.8±0.005
30	30.0±0.000	29.6±0.010

CORRELATION MATRIXES AND LINEAR REGRESSION

Correlation matrixes were gathered on the data collected at 0, 3, 24, 30, 54-, 72-, 78-, and 96-h interval to determine the relationship of the variable's concentration in ppm, OB, and the water parameters. The significantly related variables were tested by linear regression for its R, F, and p-value.

The correlation coefficients (Pearson's r) indicate the strength and direction of relationships between pairs of variables. The linear regression results provide insights into how well the predictors explain the variation in the response variable (toxicity). The result for ferric sulfate showed a strong positive correlation between concentration and DO (r = 0.925, p = .008), concentration and water hardness (r = 0.984, p = <.001), concentration and TOC (r = 0.946, p = .004), DO and TOC (r = 0.824, p = .044), water hardness and TOC (r = 0.942, p = .005), and OB and pH (r = 0.880, p = .021). The linear regression analysis suggests that concentration significantly predicts DO [R^2 = 0.851, F (1, 4) = 22.8, p = .008], concentration significantly predicts water hardness [R^2 = 0.986, F (1, 4) = 122.0, p = <.001], concentration predicts TOC [R^2 = 0.912, F (1, 4) = 12.4, p = .024], DO predicts TOC [R^2 = 0.672, F (1, 4) = 8.19, p = .044], water hardness predicts TOC [R^2 = 0.890, F (1, 4) = 12.4, p = .005], and OB predicts pH [R^2 = 0.744, F (1, 4) = 18.6, p = .021]. There is a strong negative correlation between concentration and pH (r = -0.918, p = .010), and DO and pH (r = -0.893, p = .016). The linear regression analysis suggests that concentration significantly predicts pH [R^2 = 0.843, F (1, 4) = 13.6, p = .002], and DO predicts pH [R^2 = 0.851, F (1, 4) = 22.8, p = .016].

The results taken from the ferrous sulfate water parameters suggest strong positive correlations between OB and DO (r = 0.823, p < .044), and TOC and water hardness (r = 0.981, p = <.001). There is a strong negative correlation between pH and water hardness (r = -0.869, p = .025), and pH and TOC (r = -0.912, p = .011). Several variables significantly predicted outcomes: OB significantly predicts DO [R^2 = 0.962, F(1, 4) = 101.0, p = <.001], pH predicts water hardness [R^2 = 0.756, F(1, 4) = 12.4, p = .025], and pH predicts TOC [R^2 = 0.833, F(1, 4) = 25.7, p = .011].

The water parameters in the acute toxicity test can influence how toxic substances affect fish. According to a study by Smith and Heath (1979), fish absorb toxic compounds more readily when temperatures increase. Additionally, water hardness plays a role in modifying the toxicity reaction; toxicity increases when the Ca^{2+} content is low, as Gregory and MacFarlane (1981) and Lloyd (1992) noted. Lopes and Fossum (1995) reported a positive correlation between iron concentration and total organic carbon. They also reported that organic carbon forms complexes with iron at lower pH levels. This observation suggests a plausible explanation for the observed reduction in TOC levels. Furthermore, the solubility of iron is influenced by factors such as pH, redox potential, temperature, dissolved oxygen, and other substances that act as a binding agent, as highlighted by Slaninova, Machova and Svobodova (2014). The water parameters in fishponds near blacksmith shops must be monitored as the findings suggested the effect of temperature, pH, water hardness, total organic carbon, and dissolved oxygen on toxicity as the concentration of iron increases.

During the exposure of Nile tilapia fingerlings to ferric iron, the water quality parameters that were monitored included DO (Alam et al. 2021), temperature (Dan-Kishiya et al. 2016) and water hardness (Van Aardt & Booysen 2004). The study showed that these water quality parameters remained within the acceptable range for *Oreochromis niloticus*, hence, the aforementioned water quality might not be the reason behind the decreasing operculum movement. The acidic pH might have decreased the opercular beats because at higher concentration ferric sulfate can precipitate at the bottom of the aquaria and attach at the gills resulting in prolonged stress and reduced growth (Smith, Sykora & Shapiro 1973). Figure 6 represents the network diagram between

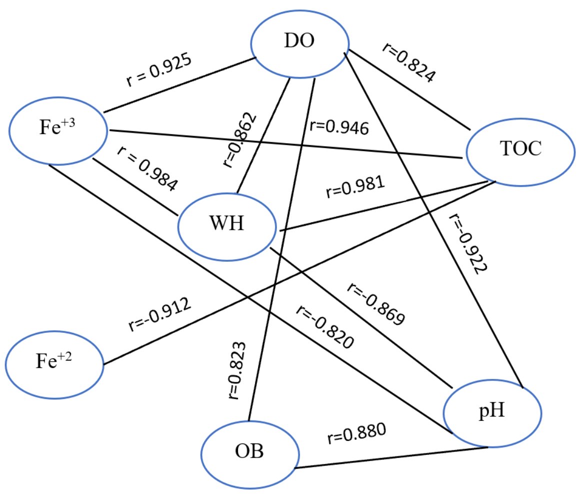

FIGURE 6. Diagram of the relationships and predictive connections of the variables

the water parameters [pH, dissolved oxygen (DO), total organic carbon (TOC), water hardness (WH)], opercular beats, and concentration of ferric (Fe^{+3}) and ferrous (Fe^{+2}) sulfate. The diagram visualizes the intricate relationships and predictive connections among the variables enabling a clear understanding of how the variables are interrelated. This diagram can guide in future research about the acute toxicity of iron sulfate to Nile tilapia. A copy of the paper was given to the City Planning Department to guide the zoning of the blacksmith shops and fishponds. Further study is being conducted by measuring the distance between the blacksmith shops and fishponds involved.

CONCLUSIONS

Ferric sulfate poses a greater risk of acute toxicity to test animals compared to ferrous sulfate, with lower lethal concentrations and shorter time to mortality. The significant difference in toxicity levels between the two substances highlights the importance of considering chemical speciation and oxidation states when assessing their environmental impacts. These results suggest that ferric sulfate may require more stringent regulatory controls and monitoring in aquatic environments to prevent its toxicity and adverse effects on aquatic organisms.

Based on the toxicity data, it is recommended to limit the use and discharge of ferric sulfate, especially in aquatic environments where sensitive organisms may be present. Insoluble ferric precipitates in the gills causing physical damage, hindering oxygen uptake, and possibly caused the decreased opercular beats and abnormal bottom distribution. Further research is needed to understand the mechanisms underlying the observed differences in toxicity between ferric sulfate and ferrous sulfate. Regulatory agencies and industries should consider the toxicity data when establishing guidelines and standards for the use and release of these substances into the environment. Risk assessments should take into account the specific environmental conditions and potential interactions with other chemicals to accurately evaluate the ecological impacts of ferric and ferrous sulfate exposure.

Overall, the acute toxicity test results emphasize the importance of assessing and managing the risks associated with ferric sulfate and ferrous sulfate exposure in aquatic ecosystems, with particular attention to the differing toxicological properties of these substances. The safety data sheets of ferric sulfate and ferrous sulfate must be followed in case of increased amounts detected in fishponds and blacksmith shops and the disposal procedure must be followed strictly.

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