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Research Article

Effects of formaldehyde bisulfite sodium on the reduction of nitrogen compounds in the tanks, hematology, and immunity of *Cyprinus rubrofuscus*

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Abstract

It is well known that ammonia nitrogen is a major pollutant in aquaculture, which can have toxic effects on fish. When absorbed in toxic concentrations, ammonia nitrogen can enter the bloodstream of fish, affecting blood parameters, immune responses, and causing oxidative damage and neurotoxicity. Recently, a study was conducted to investigate the toxic effects of ammonia on the blood, liver, growth, tissue damage, and immune indices of *Cyprinus rubrofuscus* in the presence of formaldehyde bisulfite sodium (FBS). The study involved 360 *C. rubrofuscus*, which were randomly distributed among 24 aquaria with a ratio of FBS to ammonia set at 31mg/L:1mg/L. The experiment was conducted with 15 fish in 6 treatments and 4 repetitions until 50% mortality was observed. The growth, histology, hematology, immunity, liver enzymatic and biochemical features of the fish were studied, and the results were analyzed using one-way analysis of variance (One-Way ANOVA) and Duncan's test. The study found that the blood, immune, and liver indices of koi fish changed in the presence of FBS. Additionally, adding FBS to aquarium water reduced *nitrogen* compounds in fish storage tanks, further reducing the nitrogen compounds in fish aquaria.

Introduction

Nowadays, due to the growth of population and the need for animal protein, fish has achieved a special position as a sustainable and efficient source of high-quality protein (Guo and Li, 2023). Aquaculture has grown significantly in recent decades, so much so that in 2016, the production of farmed aquatic animals reached more than 150 million tons (FAO , 2018). It reached from 183647 tons to 489205 tons from 2008 to 2018 in Iran (Statistical Yearbook of Iranian Fisheries., 2018). One of the most important issues related to growing an aquatic species is recognizing the relationship between biological and non-biological parameters and it impacts on the growth of the existing survival and determining the pattern of relationships between them (Wyban *et al*., 1986) . According to the findings, the quality of water directly affects the health and growth of farmed creatures. In farming closed circuit systems of aquatic animals, water pollution is permanently increased by farmed aquatic animals purified by purifying systems (Gök *et al*., 2009). The pollution of a closed circuit system is caused by aquatic animals and consumable (Lu *et al*., 2015).

Ammonia is the ultimate product and the main excretion matter of aquatic animal and is created by the corruption of organic materials and nitrogen compounds. Some ammonia molecules are transformed into ammonium ions in water in a reaction (Lavens and Sorgeloos, 1996) . Although ammonium is harmless for the fish, free ammonia is so toxic. According to the statistics, 0.02 mg/L is the maximum limit for Salmonidae (Sattari and Motamed, 1998) . Exposure to ammonia may have lots of toxic impacts on the fish and influence their physiological and biochemical performances. The accumulation of ammonia in the fish tissue disturbs the blood circulation system and different hematological parameters related to lipid metabolism, immune defense system, coagulation, and molecular transfer. Ammonia accumulation in the fish causes oxidative stress through the excessive production of Reactive Oxygen Species (ROS). Ammonia accumulation activates antioxidant enzymes such as *superoxide dismutase* (SOD), Catalase (CAT), and *Thiobarbituric* reactive substances (TBARS) activated in the fish to reduce oxidative stress. Besides, exposure to ammonia causes tissue damage such as the gill, liver, and kidney of the fish through oxidative damage and physiological toxicity. Briefly, fish exposed to ammonia may cause toxic effects in different systems and it is possible to use blood indices influenced by ammonia toxicity as an important parameter to evaluate ammonia toxicity in aqueous media (Xu *et al*., 2021). One of the available methods to remove ammonia in aqueous media is the use of Formaldehyde Bisulfite Sodium (FBS). Approved by the *American Food and Drug Administration*, FBS (CH_3NaO_4) is one of the compounds that can chelate water ammonia and remove it from the environment (Amit and Kenneth, 2018; FDA, 2018).

Considering the importance of ammonia toxicity in aquaculture and the problems in the identification and removal of this issue, it seems necessary to find new methods to remove fish stressor factors such as free

ammonia. Therefore, some studies have been conducted in Iran and abroad. For example, (Guo *et al*., 2023) stated that most *biological tissues* are penetrable to ammonia (against ammonium or ionized ammonia); additionally (McKenzie *et al*., 2008) stated that the highest lethal effect of ammonia is caused by influencing neurons and thus increasing N-Methyl-D-Aspartic acid (NMDA) receivers, leading to cellular death and finally the death of fish (Banihashemi *et al*. 2016) studied the histopathological effects of ammonia on the gill, kidney, and liver of *Acipenser persicus,* revealing that the histopathological damage of the gill, liver, and kidney is directly related to the increased ammonium content over time and symptoms such as hyperemia, hyperplasia, secondary bones adhesion, primary bones inflammation, cellular bleeding and necrosis in gill, hyperemia, cholestasis, cellular necrosis and hepatocytes atrophy in liver and hyperemia, interstitial cystitis, cellular necrosis, bowman space expansion, and hemosiderosis (renal deposits of iron) in the kidney. Moreover, (Mazandarani *et al*., 2016) investigated the effect of long-term challenge of unionized ammonia concentrations on the growth and hematology indices of *Rutilus caspicus*, realizing that although 0.2 mg/L molecular ammonia does not lead to the death of fish, it may be considered a harmful concentration for the growth and hematology factors of the mentioned fish. The current study has been conducted to identify the responses of *Cyprinus rubrofuscus* to ammonia chronic stress in the aquarium to evaluate its destructive effects and the potential removal of these effects with the help of FBS in this species.

Materials and methods

This is a cross-sectional-descriptive study conducted in the private workshop of ornamental fish propagation and breeding (Rasht-Guilan) for three weeks. Based on the experimental design, 24 aquaria with dimensions of 100×40×40 cm^3 and 360 *Cyprinus Rubrofuscus* with an average weight of 698±1.48 and 0.99±55.16 g were selected. Each aquarium contained 100 L of water. The fish were randomly divided into 24 aquaria (15 fish in each aquarium) after being transported to the laboratory. In this study, the diet of 21 Beyza Feed Mill Co. (Shiraz, Iran) (protein 52%, fat 12.5%, raw fiber 1.5%) was used in the amount of 2 mL for feeding. The first experimental treatment consisted of 0.75 mg/L *ammonia* and 2.325 g per100 L FBS. Besides, C- was a negative control with no additives and C+ was considered as a positive control with 4.65 g per 100 L FBS (Sigma) (to investigate the probable negative effect of this compound). During the experiment, the exposure period was determined as 12 h of light with 25 W lamps and 12 h of darkness for the aquaria. To avoid the risk of disease transmission, the aquaria were disinfected using salt and betadine, then drained and washed with 3% chloramine T solution. All aquaria were drained after 24 h and then dried. After the drying stage, the aquaria were filled with water for the first time.

Evaluation of the quality of fish and aquaculture medium

Mortalities and changes in the swimming behavior, the color of fish were recorded

and photographed in all aquaria (with and without FBS bag) during experiments. Moreover, the weight and length of the fish were measured at the beginning and the end of the experiments. The tanks were aerated using an airstone connected to the central compressor. To reduce fish stress and prevent contamination, residual food and wastes were siphoned, first day and then over time (every day at sunset after the last feeding). It is worth noting that 25-50% of the water in the tanks was exchanged once or twice a week. In case of fish mortality during the experiments, the dead fish were exited immediately.

Water parameters measurement

Dissolved Oxygen, pH, and temperature measurement

In this study, an *oximeter* (AZ 8403) was used to measure aquarium dissolved oxygen. Additionally, a digital pH meter and an aquarium thermometer were used to measure water pH and temperature, respectively.

Total hardness (TH)

It indicates the values of dissolved magnesium and calcium ions. There is a combination of calcium, magnesium, salt, and some other materials in most waters, the increase of which needs to be prevented. First temporary hardness has been formed in water, then total hardness is formed by the entrance of carbonate to water. Here, the titrimetric method or Ethylenediaminetetraacetic *acid* (EDTA) was used to measure calcium and magnesium in water.

Electrical conductivity (EC)

EC-meter was used to measure electrical conductivity. To perform this experiment, the sum of anions and cations was based on mEq/L, since they have to be equal or have insignificant differences. The greater the number of solute ions, the greater the difference in the total mentioned.

Ammonia and ammonium measurement

Nessler's method was used to determine the amount of ammonia in water using a spectrophotometer. To this end, mineral stabilizers were used to complex the hardness of the water sample. Moreover, *polyvinyl alcohol* was added to the system as a dispersing agent for the formation of color in the reaction of Nessler's reagent with ammonia. A yellow color is formed proportional to the ammonia concentration. It should be noted that experimental results are measured at 425 nm.

Growth indicators

The biomass of the fish was measured two hours before feeding. Furthermore, the growth indices were calculated using the following formulas:

$$WG = BW_f - BW_i$$

$$BWI = \frac{BW_f - BW_i}{BW_i} * 100$$

$$GR = \frac{BW_f - BW_i}{T}$$

$$SGR = \frac{lnW_f - lnW_i}{T} * 100$$

$$SR = \frac{Q_f}{Q_i} * 100$$

BWi=Average initial weight of the fish fry (g); BWf =Average final weight of the fish fry (g); Wi=Average initial biomass of the fish fry (g); Wf =Average final biomass of

the fish fry (g); TL=Total length of the fish fry in centimeters; Qi=Initial number of fish fry; Qf=Final number of fish fry; F=Amount of food consumed by the fish fry; T=Duration of cultivation (in days)

Histopathology study

To take the tissue samples of the fish on the third day, i.e. An pproximately the time of the return and release of ammonia from FBS to the water, two fish were taken from each treatment with four repetitions, totaingy 8 fish. The bag containing FBS was exited simultaneously sampling. The amount of total ammonia was measured by daily checking the time of FBS returning to the water in the aquaria. If mortalities happened sooner and when at least three fish were dead in one aquarium in a day, two lethargic or moribund fish from that aquarium were sampled. To take a sample for histopathology study of gill, skin, liver, kidney, and spleen tissues, the fish were anaesthetized in phenoxyethanol solution, then sampled and fixed using %10 buffered formalin fixatives. The standard procedure was used to prepare 5 µm tissue sections and then stained using hematoxylin and eosin (H&E) stain. The stained parts were examined by a light microscope.

Hematology study

To study the blood indices, at the end of the experiments and after the beginning of mortalities, two fish were randomly selected from repetition and treatment and the fish blood was sampled from the back of the anal fin. During the process of blood sampling, anaesthetic agents must not be used due to their effects on blood indices. After blood sampling, some blood was transferred to *Eppendorf* containing heparin (anti-coagulation material) to measure blood indices. Blood parameters such as the number of red and white blood cells, hematocrit, hemoglobin, white blood cells differential count including lymphocyte, eosinophil, neutrophil and monocyte, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration, albumin measurement, and total protein were measured by the standard methods (Torrecillas *et al*., 2011).

Measurement of C3 and C4

The immunoturbidimetric method and AutoAnalyzer (Prestige, Japan) were used to measure C3 and C4 parameters.

Complement C4 assay

An ELISA kit for Fish Complement 4 (C4) was utilized to quantify C4 in a fish serum sample. The process involved incubating C4 in a well with a monoclonal antibody enzyme coated with a monoclonal antibody specific to C4. A 40-microliter sample was combined with 10 microliters of C3 antibody and 50 microliters of streptavidin, followed by a 60-minute incubation at 37°C. Subsequently, 50 microliters of *chromogen solutions* A and B were introduced and left to incubate for ten minutes at 37°C. The addition of 50 microliters of stopping solution halted the reaction, resulting in a color change from blue to yellow, with optical absorption readings taken at 450 nm.

Complement C3 assay

An ELISA kit for Fish Complement 3 (C3) was utilized to quantify C3 levels in a fish

serum sample. The process involved incubating C3 with a monoclonal antibody enzyme coated with fish complement 3 monoclonal antibody (C3). A mixture of 40 microliters of the sample, 10 microliters of C3 antibody, and 50 microliters of streptavidin was incubated at 37°C for 60 minutes. Subsequently, 50 microliters of chromogen solutions A and B were added and incubated for ten minutes at 37 degrees Celsius. Following this, 50 microliters of stopping solution was added, causing a color change from blue to yellow, and the optical absorption was measured at 450 nm.

Statistical analysis

The data was analyzed using the Excel and SPSS 17 software packages. The comparison of the means of the treatments was carried out in the form of a completely randomized design using *one-way analysis of variance* (One-Way ANOVA) and with the help of Duncan's test. The presence or absence of a significant difference was determined at the 5 percent confidence level ($p<0.05$).

Results

Study of water indices

Water hardness

The results of water hardness on the 1st and 6th days of the study showed no difference between the treatments. According to the results in Table 1, on the first day, the highest and the lowest mean total hardness (mg/L) were related to negative control treatment (240.67) and main treatment (229.33) and again on the sixth day, the highest and the lowest mean total hardness (mg/L) were related to negative control treatment (251.33) and main treatment 2 (226), respectively. It is worth mentioning that in all aquaria, chlorine varied from 19 to 23 mg/L while water hardness varied from 226 to 251 mg/L. On the first day, the least ammonium content was reported 0.030 mg/L nitrogen in the positive control group, followed by the negative control group (0.87). The experiments were repeated twice and the p-value was $p>0.05$ in all experiments (Table 1).

Table 1: Descriptive values and total carbonate hardness (mg.L) experiment based on treatment and days of study.

	Day 1	Day 6
Treatment 3	229.33±6.03	$226±6.24^{A}$
Treatment 2		
C^{+}	238.33±7.64	$243.67±25.97^{B}$
C^{-}	$240.67±6.03^{a}$	$251.33±3.21^{C}$

Different lower and upper-case superscripts show significant difference (α=0.05) in each row and column, respectively.

pH

The results revealed no significant difference in pH between experimental treatments based on the days of study. According to the results, on the first day of the study, the highest and the lowest pH was related to the main treatment3 and negative control treatment, respectively. Also on the sixth day, the highest and the lowest pH was related to treatment1 and treatment4, respectively. The experiments were repeated twice. The p-value in all experiments was $p>0.05$ (Table 2).

Table 2: Descriptive values and pH experiment based on treatment and days of study.

	Day 1	Day 6
Treatment 3	8.24±0.08^{bB}	8.19±0.01^{b}
Treatment 1		8.13±0.01^{b}
Treatment 4		
C^+	8.12±0.09	8.18±0.02
C^-	7.7±0.2	8.15±0.02

Different lower and upper-case superscripts show significant difference (α=0.05) in each row and column, respectively.

Electrical conductivity

The results of electrical conductivity were investigated according to the treatments and based on the days of study. According to Table 3, the results revealed no significant difference in EC between study treatments and the days of study. According to the results, on the first day of the study, the highest and the lowest EC were related to the positive control treatment and treatment4, respectively. Additionally, on the sixth day, again the highest and the lowest electrical conductivity was related to positive control treatment and treatment1, treatment4, respectively. . The experiments were repeated twice and the *p*-value was $p>0.05$ in all experiments.

Table 3: Descriptive values and electrical conductivity (MS) experiment based on treatment and days of study.

	Day 1	Day 6
Treatment 4	920±5^{ab}	941±7.94^{b}
Treatment 1		931.67±2.89^{b}
Treatment 4		
C^+	934.67±5.03^{ab}	941±7.94^{b}
C^-	925±5	935±5

Different lower and upper-case superscripts show significant difference (α=0.05) in each row and column, respectively.

Temperature

The results of temperature (C) were investigated according to the treatments and based on the days of study. According to Table 4, the results revealed no significant difference in temperature between study treatments and the days of study. According to the results, on the first day of the study, the highest and the lowest temperature was related to the main treatment4 and positive control treatment, respectively. Also on the sixth day, the highest temperature was related to the main treatments 1, 2, 3 and positive control treatment and the lowest temperature was related to negative control treatment and treatment4 . The experiments were repeated twice and the *p*-value was $p>0.05$ in all experiments (Table 4).

Table 4: Descriptive values and temperature (C) experiment based on treatment and days of study.

	Day 1	Day 6
Treatment 4	19±0	19.33±0.58
Treatment 2		19.33±0.58
Treatment 3		19.33±0.58
Treatment 4		
C^+	19.67±0.58	19.33±0.58
C^-	19.33±0.58	19.00±0.00

Different lower and upper-case superscripts show significant difference (*α=0.05*) in each row and column, respectively.

Dissolved oxygen level

The results revealed no significant difference in dissolved oxygen levels (mg/L) between study days related to each treatment. According to the results, on the second day of the study, the highest dissolved oxygen level was related to positive control treatment and the lowest dissolved oxygen level was related to the main treatment4; while on the sixth day, the highest dissolved oxygen level was related

to the negative control treatment and the lowest dissolved oxygen level was related to the main treatment4. The experiments were repeated twice and the p-value was $p>0.05$ in all experiments (Table 5).

Table 5: Descriptive values and dissolved oxygen (mg.L) experiment based on treatment and days of study.

	Day 2	Day 6
Treatment4	4.49 ± 0.48^{AB}	4.4 ± 0.68^{A}
C^+	6.3 ± 1.09	6.4 ± 0.38^{AB}
C^-	6.25 ± 0.94^{AB}	7.2 ± 0.25^{B}

Different lower and upper-case superscripts show significant difference (α=0.05) in each row and column, respectively.

Ammonia and ammonium measurement

As expected, on the first day the highest amount of ammonia in terms of miligrams of nitrogen per liter 1.67 in the positive control group was reported 0.03 and by following of the negative control group it was repoted negative. The amount of ammonia on the sixth day in group 4 was almost double that it was approximately six to eigh times that of the first three groups (Table 6).

Table 6: Descriptive values and NH_4 (mg L) experiment based on treatment and days of study.

	Day 1	Day 6
Treatment 1	98.0 ± 0.08^{Bb} 0.89 ± 0.01^{Bb}	0.32 ± 0.02^{Aa} 0.35 ± 0.02^{Aa}
Treatment 2	1.22 ± 0.22^{Cc} 1.67 ± 0.16^{Dbc}	0.39 ± 0.02^{Aba} 2.43 ± 0.05^{Cc}
Treatment 3		
Treatment 4		
C^+	0.81 ± 0.25^{b}	0.20 ± 0.01^{Aa}
C^-	0.03 ± 0.00^{Aa}	1.12 ± 0.01^{Bb}

Different lower and upper-case superscripts show significant difference (α=0.05) in each row and column, respectively.

Growth indicators

Body weight increasing indicator

The highest average initial weight (g) related to treatment 4 had no significant difference from other groups ($p<0.05$). At the end of the experiments and based on the results, the lowest value of secondary weight (gram) was related to group 4, only showing a significant difference from the positive control group ($p<0.05$) (Table 7).

Table7: Descriptive values and dissolved WG (g) experiment based on treatment and days of study.

	WG (g)
Treatment1	1.19 ± 0.2^{c}
Treatment2	0.4 ± 0.3^{a}
Treatment3	0.55 ± 0.75^{a}
C^+	1.47 ± 0.47^{d}
C^-	1.19 ± 0.2^{c}

Different lower and upper-case superscripts show significant difference (α=0.05) in each row and column, respectively.

Growth rate indicator

Based on the available data, the highest percentage of increase in body weight elsted to group 1 with a value of 1.19% without any significant difference ($p<0.05$) from group 6 or the same negative control group. These rates of increase in groups 2 and 3, which were exposed to higher ammonia contents, were calculated as 0.4% and 0.55%, respectively. Meanwhile, the percentage of body weight increase in the positive control group is 1.47%, significantly higher than other groups ($p<0.05$) (Table 8).

Table 8: Descriptive values and dissolved BWI (%) experiment based on treatment and days of study.

	initial weight (g)	secondary weight (g)
Treatment4	48.58 ± 0.26	48.12 ± 0.45^{a}
C^+	48.26 ± 0.26	48.96 ± 0.14^{b}
C^-	48.14 ± 0.23	48.71 ± 0.28^{ab}

Different lower and upper-case superscripts show significant difference (α=0.05) in each row and column, respectively.

Specific growth rate indicator

Based on the available data, the lowest and highest specific growth rates are -0.032 and 0.048 in groups 4 and 5, respectively. The highest specific growth rate in the 4 treatment groups is related to group 1 with a value of 0.039 with no significant difference from group 6 (Table 9).

Table 9: Descriptive values and dissolved SGR (%) experiment based on treatment and days of study.

	SGR (%)	
Treatment4	-0.032±0.04	0.039±0.007
Treatment1		
C^+	0.048±0.016	
C^-	4.28 ± 35.025^{a}	

Different lower and upper-case superscripts show significant difference (α=0.05) in each row and column, respectively.

Survival rate indicator

The highest survival rate among the treatments is 83.22% in group 1, without a significant difference from group 2 (82.22%). It is worth noting that the lowest survival rate is 77.78% in group 3. The survival rate of the positive control group with 4.65g of FBS was also 100%, with no significant difference from the negative control (100%) (Table 10).

Table 10: Descriptive values and dissolved SR (%) experiment based on treatment and days of study.

	SGR (%)	
Treatment1	83.22±16.78 b	0.039±0.007
Treatment2	2.22±10.18 b	
Treatment3	77.78±10.28 a	
C^+	100±0 c	
C^-	100±0 c	

Different lower and upper-case superscripts show significant difference (α=0.05) in each row and column, respectively.

Investigation of blood indices

Hematology

According to Table 11, the results of these investigations showed a significant difference between treatments in lymphocyte index so that the highest and the lowest percentage of mean lymphocyte index was related to the main treatment 2 (83.5) and negative control treatment (78.33), respectively. Additionally, a significant difference was observed between treatments in the *neutrophil* index, i.e. The highest and the lowest percentages of the mean neutrophil index were related to the negative control treatment (16.33) and main treatment (12.33), respectively. The experiments were repeated twice and the p-value was $p>0.05$ in all experiments (Table 11).

Table 11: The differences of blood indices based on study treatment.

Index blood Index table	Treatment[2]	Positive control	Negative control
Eosinophils (%)	1±0	0.6 ±1.3	1±0
Monocytes (%)	3.8±0.9	4.7 ±0.8	5±0.6
Lymphocytes (%)	83.5± 2.1 b	80.3±2.5 ab	78.3±2.4 a
Neutrophils (%)	12.3±1.2	14.3±1.4	16.3±1.8
MCHC[1] (g.l)	233.4 ± 0.7	236.6 ± 0.5	232.4± 0.4
MCH[2] (pg.cell)	65.4±2.2	66.0± 1.3	65.2±0.7
MCV[3] (FL)	279.2 ± 4.9	281.2±7.1	278.5 ± 2.4
HCT[4] (%)	33± 2.4	34.2± 1.5	35.2±1.7
Hb[5] (g.dl)	7.7±0.4	8.1±0.2	8.2±0.4
RBC[6] (10^6.mL)	1.20±0.000	1.21±0.0046	1.27±0.057
WBC[7] (10^3.mL)	3.633±0.33	5.483±0.70	7.116±0.32

[1]Mean Corpuscular Hemoglobin Concentration
[2] Mean Corpuscular Hemoglobin
[3] Mean Corpuscular Volume
[4] Hematocrit
[5] Hemoglobin
[6] Red Blood Cells
[7] White Blood Cells

Measurement of blood biochemical parameters

As per Table 12, the Complement C3 index treatments exhibited a significant variation. Specifically, treatment showed the highest average, while the negative control treatment had the lowest. The Complement C4 index also revealed a significant difference among the treatments. Treatment ٤had the highest Complement C4 index, whereas negative control had the lowest. Furthermore, the albumin index differed significantly across the treatments. Treatments 2 and 3 had the highest albumin index, while treatment 1 had the lowest., Also, a significant difference between the treatments in thetotal protein index (g/dl) (p<0.001) that the highest and the lowest mean albumin index (g/dl) respectively (g/dl) was observed in treatments 2 and 3 (1.3). and the lowest mean albumin index (g/dl) was observed in treatment1 (0.9).

Table 12: The homogeneity of variance of blood serum indices experiment.

Group	C_3(mg.dl)	C_4(mg.dl)	Total protein(g.dl)	Albumin(g.dl)
T_1			1.7±0.1[a]	0.9±0.1
T_2			2.3±0.07[c]	1.3±0.03[b]
T_3			2.3±0.13	1.3±0.03[b]
T_4	98.5±5.9[ab]	46.4±1.٥[ab]		
C_+	68.5±5.9[a]	14.6±0.8[a]	1.95±0.0[b]	1.01±0.1[a]
C	62.22±3.9[b]	16.9±1.1[b]	2.2±0.1[c]	1.2±0.07[b]

These numerical details highlight the significant differences observed among the treatments.

The presence of *FBS* significantly impacts the blood serum indices of fish. Specifically, it influences the Complement C3 and C4 indices, as well as the albumin index. The presence of FBS significantly affects the blood serum indices of fish. In particular, C3 and C4 indices affect complement as well as albumin index.The highest averages for both Complement C3 and C4 were observed in treatment 3, while the lowest were seen in the negative control and positive control 1 treatments, respectively. For the albumin index, the highest average was found in treatments 2 and 3, and the lowest in treatment 1. and for total protein index highest average was fond in treatments 2 and3 and the lowest in treatment 1. These findings suggest that FBS plays a crucial role in the physiological health of fish, potentially affecting their growth and overall wellbeing.

Analysis of histopathology findings

The results of the histopathological study of different tissues depend s on the added ammonia concentrations are shown in Figures 1 to12.

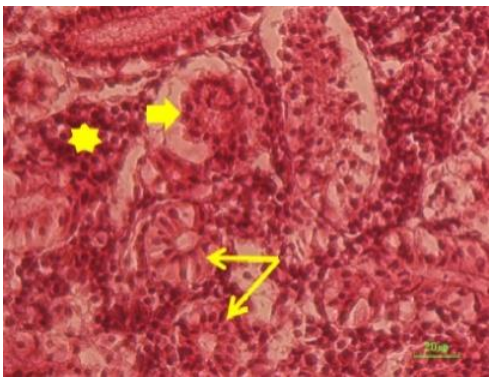

Figure 1: Fish kidney negative control. Glomerule (short arrow), urethra (long arrow) and interstitial tissue (star) are observed without illness (H&E, Bar=20µm).

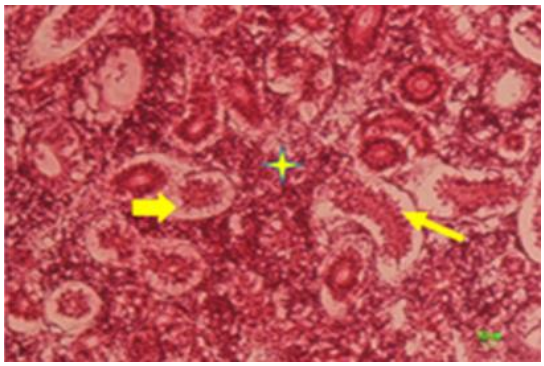

Figure 2: Negative control, fish kidney and glomerule (short arrow), urethra (long arrow) and interstitial tissue (star) are observed without illness (H&E, Bar=15µm).

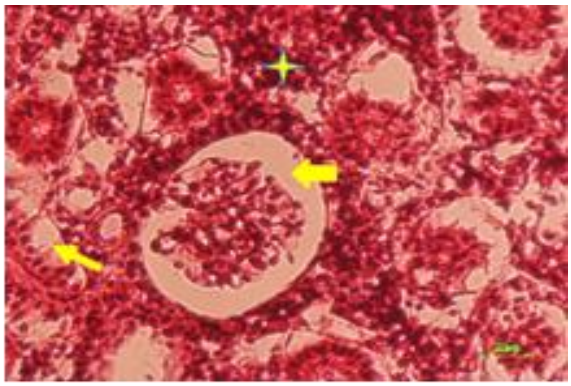

Figure 3: Negative control and kidney glomerules (short arrow), urethra (long arrow) and interstitial tissue (star) are observed without illness (H&E, Bar=25µm).

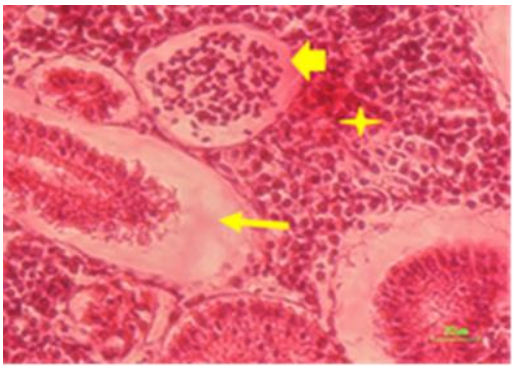

Figure 4: Kidney positive control and fish kidney glomerules (short arrow), urethra (long arrow) and interstitial tissue (star) are observed without illness (H&E, Bar=25µm).

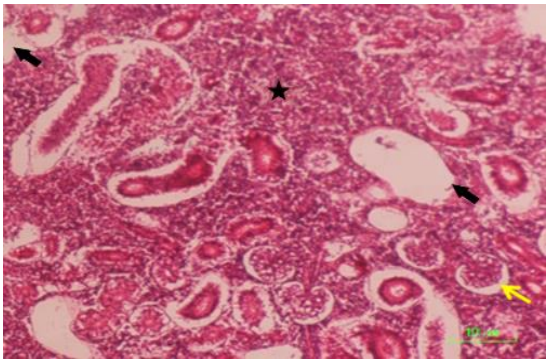

Figure 5: Kidney main treatment, glomerule bowman space expansion (short arrow) and urethra necrosis (long arrow) and also hyperemia observed in interstitial tissue (star) (H&E, Bar=10µm).

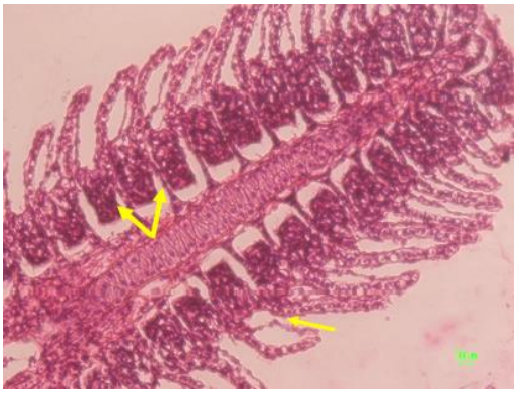

Figure 6: Main treatment of secondary strands hyperplasia gill (double arrow), edema of base layer in secondary strands (short arrow) (H&E, Bar=10µm).

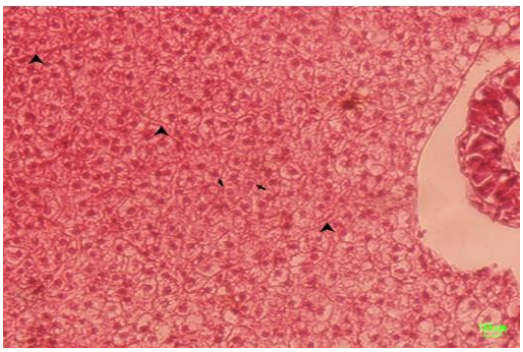

Figure 7: Negative control, liver tissue, liver normal tissue of hepatocytes (arrow) and sinusoids (tip of arrow) which are observed normal (H&E, Bar=10µm).

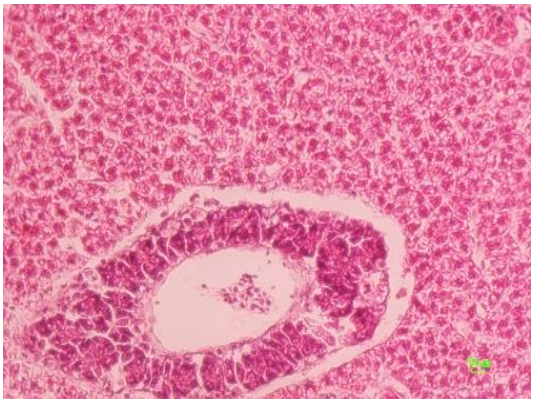

Figure 8: Liver main treatment, degeneration (short arrow), hepatocytes vacuole (long arrow) (H&E, Bar=10µm).

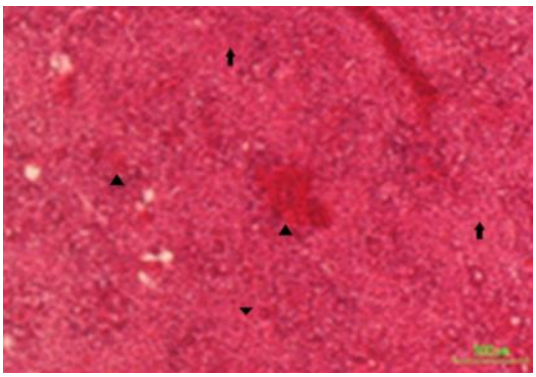

Figure 9: Negative control, fish spleen with white (arrow) and red (triangle) pulp observed without illness, (H&E, Bar=15µm).

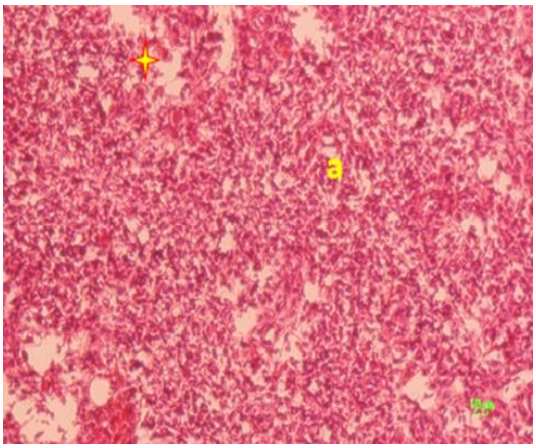

Figure 10: Spleen main treatment, red pulp hyperemia (star) and some degeneration observed in white pulp (a) (H&E, Bar=10µm).

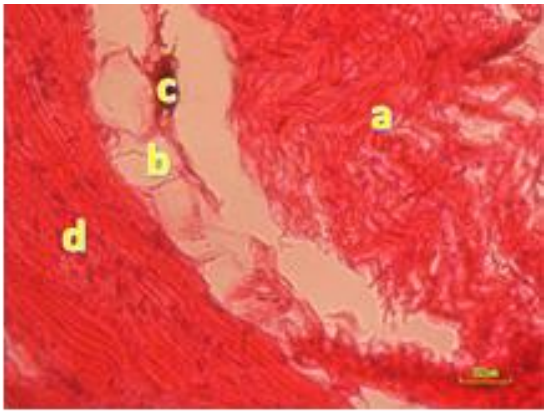

Figure 11: Negative control and fish skin, dermis (a), hypodermis (b) (c) and muscle (d) observed normal, (H&E, Bar=21µm).

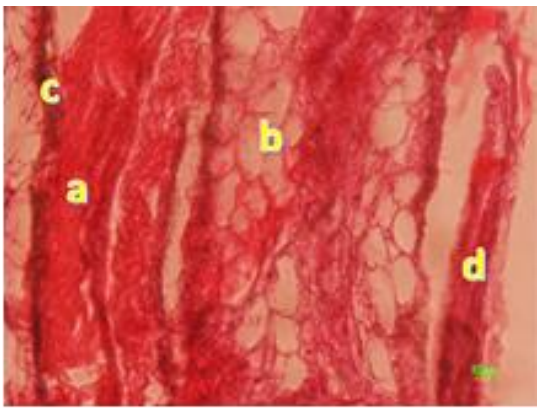

Figure 12: Negative control, fish skin, dermis (a), hypodermis (b), pigment (c) and muscle (d) observed normal, (H&E, Bar=15µm).

Discussion

About 60%-90% of the nitrogenous material secreted through the gills of fish is caused by ammonia (NH_3) and (NH_4), and 9%-27% by urea. Other ammonia resources present in aquaria and swimming pools are caused by the nitrification of nitrogen materials by microbes (Grommen *et al.*, 2002). LC_{50} or acute lethal concentration of 50% ammonia (mg nitrogen per L aqueous medium ammonia) is too high for resting fish (LC_{50}=207.0) rather than the swimming ones (LC_{50}=32.0) (Randall and Tsui, 2002). The concentration of this material shows that despite the increasing density of the fish in aquaculture systems

due to the immobility of the fish, the lethal concentration of ammonia may be much higher. Therefore, increasing fish activity in high values of ammonia is more lethal.

Growth experiments with Topeka shiners showed that the concentration of the Lowest Observed Effect Concentration (LOEC) of total ammonia for shiners of Cypriniformes order (eastern shiners are used as model fish) is 17.93 mg/L. For this fish, the Non-Observed Effect Concentration (NOEC) and the Maximum Acceptable Toxicant Concentration (MATC) have been reported around 8.05 and 12.01 mg/L, respectively. At 12.4°C temperature, these values have been reported 6.65 and 10.0 mg/L, respectively. In Fathead minnow (*Pimephales promelas*), MATC has been reported 29.12 mg/L at 24°C temperature in the challenge with ammonia (Adelman *et al*., 2009). Ammonia is found in pools or aquariums usually as ionized NH_4 and unionized NH_3 forms and total ammonia refers to the sum of both forms. Ammonia in pools or aquariums is usually found as ionized NH4 and ionized NH3, and total ammonia refers to the whole amount of both forms (Grommen *et al*., 2002).

According to a study, the chronic (constant) challenge with total ammonia as 2 mg/L during larva stages causes toxicity (CSPP[1], 2013). On the other hand and based on a general rule, the maximum acceptable limit of nitrogen materials such as total ammonia and nitrite is around 10% of LD_{50} or LC_{50} 96 h (Molayemraftar *et al*., 2022). Therefore, it was revealed that the acceptable concentration of ammonia is 51.24 mg/L in north common carp. The findings resulting from the negative control group are consistent with the results of FBS effect on the positive control group so that positive control values on different sampling days are significantly lower than negative control values on similar days, indicating suitable removing ammonia by FBS.

According to the results, it is concluded that FBS existing in main treatment bags can maintain absorbed ammonia for 2 to 6 days so the value of ammonia in this treatment, unlike the negative control group, does not change significantly from day 2 to day 6 ($p>0.05$). These results may completely confirm the role of FBS in the reduction of ammonia content. In ammonia lethal concentration for common carp (150 mg/L total ammonia content), using 10 mg/L natural zeolites prevents fish mortality (Peyghan and Takamy, 2002). In a histopathological study on the effect of total ammonia content, the most important changes observed in the gills were hyperemia, edema, and aneurysm. In the kidney, degenerative necrosis in glomerulus, bowman capsule expansion, hyperemia, congestion, and hemorrhage were the most distinguished changes. Hyperemia, degeneration and the existence of some necrosis regions were observed in the liver. The maximum value of the total ammonia tolerable for non-salmonids such as common carp, Clupeiformes, and zebrafish has been 10.0 based on mg N/l in pH: 8.1 (Randall and Tsui, 2002).

At a pH of about 7.6, (Edwards *et al*., 2024) has considered the safe total ammonia

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content about 2 mg/L and the dangerous content about 8 mg/L. Since the pH of aquarium water ranges between 7.6 and 7.8, it seems that water ammonia has been safe for the fish *(*Peyghan and Takamy, 2002*)*. According to reports, with the increase of pH from 6.5 to 8, the toxicity of ammonia is reduced and 96-h LD_{50} has increased (0.82 to 1.1 mg/L). The safe value of ammonia has been reported as less than 1.5 mg/L (Crab *et al*., 2007). According to the studies conducted on the effect of nitrite on different fish, there is a relationship between pH, water hardness, and chlorine ion, so the LC_{50} value was 12.0 mg/L in a 4-day study on rainbow trout, at the high hardness and when water hardness and chlorine ion were more than 15 mg/L and pH was more than 8.0, it has reduced to 0.5 mg/L when water hardness and chlorine ion reduced 10 times (Alabaster and Lloyd, 2013), demonstrating the effect of these three factors on increasing the level of LC_{50}, especially in alkali conditions. It is obvious that the carp family is most resistant to nitrite (Kroupova *et al*., 2010). This study has reported this value for the common carp around 40 in alkali conditions (Alabaster and Lloyd, 2013).

A hematological study showed that WBC, Hb, Hct, and RBC values significantly reduced, consistent with hematological changes reported by other researchers (Shin *et al*., 2016). They understood that MCV and MCH values have significantly reduced, although, in the current study, no significant difference was observed in their values in challenge with different ammonia contents. The aggregation of nitrogen is a serious environmental problem in freshwater basins, which may lead to the high mortality of fish and shrimps and eutrophication (Chen *et al*., 2017). A consortium consisting of three bacterial isolates, namely *Bacillus cereus*, *Bacillus amyloliquefaciens*, and *Pseudomonas stutzeri*, has been identified and collected for the biological decomposition of ammonia and nitrite in aquaculture. This consortium is used as a bioaugmentation factor in tanks containing tilapia fingerlings. During the 15 days of the experiment, under controlled temperature and light conditions, the values experimented in both treatment and control tanks were increased. The residual ammonia content in the tanks treated by 4.8 mg/L consortium was always less than the 7.2 mg/L content in control tanks without any bacteria. Increased residual nitrite and nitrate contents in the treatment tanks (6.9 mg/Land 4.16 mg/L, respectively) compared to the control tanks (0.28 mg/Land 0.394 mg/L, respectively) confirmed the efficiency of the consortium to convert ammonia to further nitrite and the less toxic nitrate (John *et al*., 2020). They found out that due to the transformation of ammonia to nitrite by oxidants, rapid reduction of nitrite content was not observed in five days. However, the rapid increase of nitrate content in purified water suggests the transformation of nitrite to nitrate with the action of oxidants. Investigations show that the results of the studies by (John *et al*. 2020) on ammonia are significantly higher than the results of our research (approximately 1.5 times). On the value of nitrite content, only their results have been equal to the six-day results of group 4 in this study;

however, in other groups, the results of this study have been less. The high value of nitrite content of this study compared to the results of John *et al*. (2020) shows the high primary ammonia content poured into the system, probably produced in the performance of oxidants in the transformation of nitrite to nitrate due to environmental changes such as temperature, etc. In different time intervals.

Like phytoplankton, periphytons are found almost in every type of water from small ponds to huge oceans and in different nutritional conditions from the most oligotrophic to the most eutrophic. Periphytons averagely have C/N ratio of 10 (Azim *et al*., 2004). The capacity of nitrogen materials absorption is around 0.2 g nitrogen m^2 (Crab *et al*., 2007) per day, but their industrial and development application has not been reported. In another study, Grommen *et al*. (2002) used Ammonia Binding Inoculum Liquid (ABIL) as a suspension to reduce the initial time of biofilters. They showed that using this solution in a 5 mg dosage of ABIL volatile suspended solids might remove total ammonia and nitrite contents from the aquatic environment of the aquarium to 10 mg/L (Grommen *et al*., 2002).

In another innovation, nitrite removal was considered to regulate the C/N ratio and infrastructural mechanisms. Nitrite removal system included 530 ml medium containing 5 mg/L NO_3-N and 66.6-0 mg/L chemical oxygen demand (COD) (for example, zero to 13.3 C/N ratio) and 20 g pool sediments. When the C/N ratio was more than 8, the efficiency of nitrite removal during the study reached 100% and the aggregation of nitrite was negligible around zero; however, the efficiency of nitrite removal was less and nitrite aggregation was signed up to 0.8 mg/L when the C/N ratio was below 8. In this study, eight types of bacteria Dechloromonas, Azoarcus, Azospira, Rubrivivax, Thiobacillus, Vogesella, and Zoogloea were identified as nitrogen oxidants. Dechloromonas was dominant among them. In ammonia lethal concentration for common carp (150 mg/L total ammonia) using 10 g natural zeolite prevented fish mortality (Rong chen *et al*, 2017). They also found that hyperemia, edema, and the aneurysm were the key damages in the histopathological changes observed in the gill.

In gill histopathology in the main treatment of secondary lamellae hyperplasia, 50% of samples showed edema of the base layer. It seems that in challenges longer than 24 h, more changes such as hyperplasia of basal cells of secondary lamellae in Peyghan and Takamy's research are not impossible. In the kidney histopathology of Peyghan and Takamy's study, the degenerative changes of tubules and glomerulus, bowman capsule space expansion, hyperemia, congestion, and hemorrhage were among the most distinguished changes (Peyghan and Takamy, 2002).

According to the references, the high albumin content in the treatment group compared to the control group can be caused by the body's need to repair damaged tissues and increased immune responses, and the increase in ammonia up to 1 mg/L increases complements C3 and C4 in the treatment group 3; however, its value decreases in treatment group 4. This

tissue damage in fish of this group may be due to high ammonia content. It seems that due to the long-lasting effect of damage in kidney tissue on fish life, the survival rate (100%) of Peyghan and Takamy's research within 24 h is not in line with our research, where most evaluations were performed after 4 to 6 days.

In the histopathological study of the kidney in treatment group 2, bowman space expansion, tubule necrosis, and hyperemia were observed in the interstitial tissue. Peyghan and Takamy (2002) reported hyperemia, degeneration, and some necrotic regions as the only damages in the kidney of *Cyprinus Rubrofuscus*. Moreover, they reported hyperemia, degeneration, and some necrotic regions as the only changes observed in the liver. The reason for this difference may be the longer-term experiment of this study than the above-mentioned research. In this study, treatment 2 showed less degeneration and hepatocyte vacuole.

Conclusions

This study demonstrated the effectiveness of formaldehyde bisulfite sodium (FBS) in reducing ammonia levels in goldfish tanks. However, the observed alterations in blood indices and liver enzymes suggest potential negative health impacts on the fish. Further research is warranted to investigate the long-term effects of FBS on goldfish health, including potential dosage optimization and potential mitigation strategies for any adverse effects.

The study demonstrates that the use of formaldehyde bisulfite sodium (FBS) can effectively reduce the concentration of toxic ammonia nitrogen in aquaculture tanks. The application of FBS at a ratio of 31 mg/L to 1mg/L of ammonia was found to significantly mitigate the harmful effects of ammonia on the blood, liver, and immune system of *Cyprinus rubrofuscus*.

Notably, the presence of FBS led to observable changes in the blood, immune, and liver indices of the fish, indicating a potential protective effect against ammonia-induced toxicity. Furthermore, the addition of FBS to the aquarium water was associated with a reduction in nitrogen compounds in the fish storage tanks, further contributing to a safer and healthier environment for the fish. These findings suggest that FBS could be a valuable tool in the management of ammonia pollution in aquaculture, potentially improving the health and survival of fish in these settings. Future research should continue to explore the optimal use of FBS and other similar compounds in different aquaculture scenarios to maximize their benefits.

Conflicts of interest

The authors declare no conflicts of interest.

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