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Research on the Toxic Effects of Water Treatment By-Product N-Nitrosodimethylamine (NDMA) on Juvenile Crucian Carp

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Abstract

N-nitrosodimethylamine (NDMA) is a relatively toxic and environmentally persistent carcinogen, posing potential threats to the sustainability of freshwater ecosystems, but relevant research on the toxic effects of NDMA in China is relatively scarce. Taking juvenile crucian carp, a key economic and ecological native fish species in China, as the experimental organism, this study explored the acute toxicity and oxidative stress effects of NDMA through semi-static acute toxicity tests combined with 5-day exposure experiments, determining the protein content, superoxide dismutase (SOD), and catalase (CAT) activities in fish. The study provides a reference for ecological risk assessments and pollution control of NDMA in freshwater environments. Results showed that NDMA exerted significant acute toxicity to juvenile crucian carp, with 24, 48, 72, and 96 h half lethal concentrations (LC_{50}) of 9.83, 9.14, 8.11, and 7.05 mg/L, respectively, and a safe concentration (SC) of 0.705 mg/L (classified as medium toxic). NDMA exhibited distinct concentration- and time-dependent effects on the indicators. Protein content showed a consistent induction effect. Low concentrations (1.18, 1.76 mg/L) induced SOD activity, while high concentrations (3.68 mg/L) significantly inhibited it. CAT activity was mainly induced but inhibited in the 3.68 mg/L group at 3–5 days. The inhibition rate order of NDMA on the indicators was SOD > CAT > protein content. This study, through the aforementioned exploration, further supplements domestic toxicological research on NDMA regarding native freshwater fish, clarifies the key acute toxicity parameters and physiological-biochemical response patterns of NDMA on juvenile crucian carp, screens SOD and CAT as potential biomarkers for NDMA pollution monitoring in freshwater environments, and also provides basic data support for the sustainable management of disinfection by-products.

Keywords: acute toxicity; oxidative stress; antioxidants; fish; water pollution

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1. Introduction

Water resources are the fundamental material for human survival and development, and safe drinking water is essential for public health [1,2]. However, rapid industrialization has aggravated water source pollution with rising organic matter content, making water disinfection indispensable for removing pollutants and pathogens. This process relies on the combined effects of physical adsorption/interception, chemical oxidation/decomposition, and biological inhibition of microbial metabolism by disinfectants [3,4].

Chemical oxidants (e.g., chlorine, chloramine, ozone) used in water treatment react with nitrogen-containing organic matter (e.g., dimethylamine) via nitrosation/oxidation to

form toxic nitrogenous disinfection by-products (N-DBPs) [5]. Over 70 N-DBPs have been detected in drinking water, among which N-nitrosodimethylamine (NDMA) is the most prevalent, with the highest concentration. NDMA exhibits strong cytotoxicity, genotoxicity, and carcinogenicity [6,7]. For instance, it can induce liver cell apoptosis and DNA strand breaks. At a concentration of only 0.7 ng/L in drinking water, NDMA may result in a lifelong cancer risk of up to 10^{-6}, and it is classified as a Group 1 carcinogen by IARC [8]. Chloramine disinfection, widely adopted since the late 20th century to reduce traditional DBPs (e.g., trihalomethanes) per international regulatory requirements, is a major source of NDMA. Historical monitoring showed high NDMA levels (up to 100 ng/L in Canadian pipe networks, 78.9 ng/L in Shanghai's 2009 pipeline water), far exceeding the 9–10 ng/L limit [9]. Recent data indicate a declining but notable trend (10–18 ng/L), with temporary surges (40.9–72.5 ng/L) during the COVID-19 pandemic due to increased disinfectant dosage [10,11].

NDMA formation is associated with nitrogen sources, dissolved organic compounds (DOC), and disinfectant types/concentrations, and it has two main generation pathways: reaction of dimethylamine (DMA) with nitrite in the environment (see Figure 1a), and in-situ production during chloramination (see Figure 1b) [12]. With high water solubility and poor removability, NDMA persists in surface and groundwater, threatening aquatic organisms across all trophic levels [13]. Current NDMA toxicity studies focus mostly on mammals, algae, and foreign aquatic organisms [14,15]. Studies on NDMA toxicity in non-native fish have shown key effects. Zebrafish exposed to sub-lethal concentrations (1–10 mg/L) exhibited oxidative stress (elevated MDA, reduced SOD/CAT) [16], while tilapia and common carp suffered histopathological damage (hepatocellular vacuolation, gill lamellae fusion) and genotoxicity (DNA breaks, altered p53/bax expression) [17]. However, these studies focus on model/non-native species, with scarce data on toxicological mechanisms in Chinese native freshwater economic fish (e.g., crucian carp) under chronic or environmentally relevant exposure.

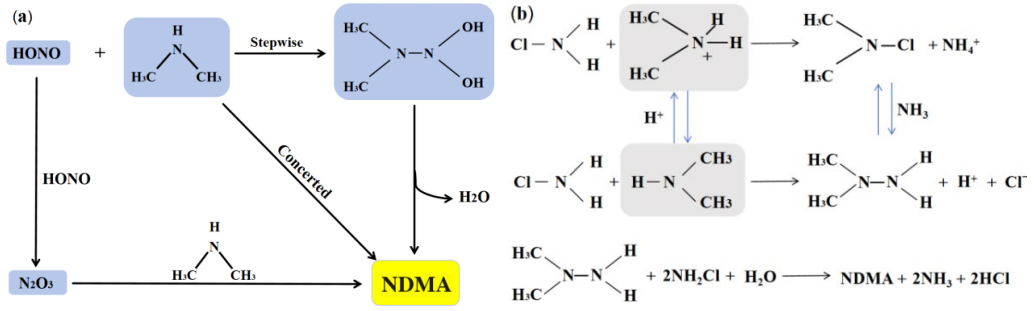


Figure 1. NDMA formation mechanism (**a**). DMA and nitrite direct reaction and indirect reaction (**b**). Formation of monochloramine in reaction with DMA.

To make up for these deficiencies, this study selected juvenile crucian carp—an economically important, widely distributed freshwater fish in China with strong environmental adaptability—as the model organism [18]. Juvenile fish were chosen for their high sensitivity to toxins, controllable experimental conditions, and low breeding costs [19]. We investigated the effects of NDMA concentrations on juvenile crucian carp's mortality, protein content, and the activities of antioxidant enzymes (SOD, CAT), aiming to clarify NDMA's toxic effects on freshwater non-target organisms and provide reference data support for environmental protection and toxicity risk assessments.

2. Materials and Methods

2.1. Animal Procurement and Maintenance

The experimental organisms were juvenile crucian carp purchased from an aquaculture base in Suzhou City. The average body length was (2.50 ± 0.50) cm, and the average weight was (0.20 ± 0.15) g. The fish were cultivated in special plastic buckets filled with dechlorinated tap water under continuous aeration (aeration time ≥ 48 h before use). Before the experiment, the fish were acclimatized to the laboratory environment for 10 days. During the acclimation period, the water was renewed daily to maintain water quality stability. The fish were fed a commercial fish diet once daily at a fixed time at a feeding rate of 2–3% of the total body weight of the fish, and any dead individuals and residual feed were promptly removed to avoid water pollution. The mortality rate was strictly controlled to be less than 2% during the acclimation period. Only healthy individuals of uniform size, intact body shape, and agile movement were selected for the formal experiment. Feeding was stopped 24 h before the start of the experiment to eliminate the interference of food digestion on the experimental results. The water quality parameters during acclimation were maintained as follows: water temperature 26.5 ± 1.5 °C, pH 7.5 ± 0.06, conductivity 135 μs/cm, dissolved oxygen 7.6 ± 0.4 mg/L, total hardness 49.0 ± 3.0 mg/L, copper < 0.009 mg/L, mercury < 0.00004 mg/L, lead < 0.002 mg/L, chromium < 0.004 mg/L.

2.2. Test Instruments and Reagents

In this experiment, the NDMA was detected by headspace solid-phase microextraction gas chromatography-mass spectrometry [20]. The chromatographic column selected was an Agilent HP-5 series elastic quartz capillary column, with a specification of 30.0 m long × 0.25 mmI.D, and df = 0.25 μm. We used the following column box heating process: initial temperature 40 °C for 2 min, then temperature to 120 °C (rate: 10 °C/min) for 1 min, then temperature to 200 °C for 8 min, and finally temperature to 300 °C (rate: 20 °C/min) for 1 min. The flow rate of carrier nitrogen and helium was 1 mL/min. The chromatography detector has an ionization potential of 70 eV, an inlet temperature of 250 °C, and the detection was in ion mode (SIM).

Instruments: GC-MS Gas Chromatography-Mass Spectrometry, model 7890 (Agilent Technologies, Santa Clara, CA, USA); UV visible spectrophotometer, model UV-2601 (Beijing Beifen Ruili Analytical Instrument (Group) Co., Ltd., Beijing, China); Handheld electric speed control grinder, SC-50 (Beijing Xianfeng Keyi Instrument Co., Ltd., Beijing, China); Thermostatic water bath pot, HH-6 (Jintan Honghua Instrument Factory, Changzhou, Jiangsu, China); High speed freezing centrifuge, H1750R (Hunan Xiangyi Laboratory Instrument Development Co., Ltd., Changsha, Hunan, China); Ultrasonic cell grinder, SCIENTZ-ID (Ningbo Scientz Biotechnology Co., Ltd., Ningbo, Zhejiang, China); Pure water/ultrapure water device, Zrxq0150 (Zhejiang Quanwo Environmental Protection Technology Co., Ltd., Hangzhou, Zhejiang, China); Microbalance, XPR2U/AC (Mettler Toledo, Columbus, OH, USA).

Test reagents: N-nitrosodimethylamine, ethylenediaminetetraacetic acid (EDTA), ammonium molybdate, disodium hydrogen phosphate, tris(hydroxymethyl)aminomethane (Tris) (analytically pure, purchased from Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China); sodium dihydrogen phosphate, sodium chloride (analytically pure, purchased from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China); bovine serum albumin, Coomassie Brilliant Blue G-250 (purchased from Shanghai Sangbao Bio-Tech Co., Ltd., Shanghai, China); anhydrous ethanol (95%, analytically pure, purchased from Wuxi Jingke Chemical Co., Ltd., Wuxi, Jiangsu, China); pyrogallol (analytically pure, purchased from Chengdu Prysmian Bioscience Co., Ltd., Chengdu, Sichuan, China). All biochemical

indices in this study were determined using commercial assay kits, including a BCA Protein Assay Kit (purchased from Beyotime Biotechnology, Shanghai, China), as well as an SOD Activity Assay Kit and a CAT Activity Assay Kit (both purchased from Suzhou Jinertai Laboratory Equipment Co., Ltd., Suzhou, Jiangsu, China).

2.3. Test Methods

2.3.1. Acute Toxicity Test of NDMA on Juvenile Crucian Carp

This study first conducted a pretest: In the experiment, five high-concentration and five low-concentration toxic solutions were used, with ten concentration groups and one control group setup, and three parallel experiments were conducted in each group. Using a semi-static experimental method (replacing 50% of the exposure solution with fresh solution of the same nominal concentration every 24 h to maintain the stability of NDMA concentration, pH and water temperature, continuously aerating to ensure sufficient dissolved oxygen, and removing feces and residual bait in a timely manner), a 96 h exposure test was conducted on juvenile crucian carp, and the lowest concentration of all deaths within 24 h (LC_{100}) and the highest concentration of no deaths within 96 h (LC_0) were obtained to determine the suitable concentration gradient range for the formal test [21]. Based on the concentration range results from the pretest, a formal test was conducted. Eight treatment groups were designed using an equal logarithmic spacing method, which allows concentrations to increase proportionally across a wide exposure range, ensuring appropriate coverage of both low- and high-toxicity levels and improving the accuracy of LC_{50} estimation in acute toxicity tests. The acute toxicity test was conducted for 96 h. For each formal treatment group, 20 healthy juvenile crucian carp were selected and evenly allocated to four parallel 500 mL beakers (5 fish per beaker). Each beaker was filled with 500 mL of the corresponding concentration exposure solution. The half lethal concentration (LC_{50}), safe mass concentration (SC), and toxicity accumulation coefficient (MAC) of NDMA on juvenile crucian carp were obtained.

2.3.2. Toxicity Test of NDMA on Physiological and Biochemical Indexes of Juvenile Crucian Carp

Based on the results of the acute toxicity test, four concentrations were selected as the experimental group for poisoning, and a blank experimental group was set as the control to study the changes in protein content, superoxide dismutase (SOD), and catalase (CAT) activity in the bodies of juvenile crucian carp under different concentrations of NDMA stress to evaluate the toxic effects of NDMA on relevant indicators in the bodies of juvenile crucian carp [22,23]. A 5-day (120 h) exposure duration was adopted in this assay, which is sufficient to capture the time-dependent cumulative changes in SOD, CAT, and protein content in fish under NDMA stress. This duration allows for the effective characterization of subacute toxic responses of the fish antioxidant defense system, which aligns with the core objective of this study to investigate physiological and biochemical toxic effects.

For the biochemical assays (SOD, CAT, and protein content), the tissue source was whole bodies of juvenile crucian carp. Two juvenile crucian carp were taken from each concentration group and the blank control group during the exposure period. The fish were killed at low temperature, and the surface contamination solution was blotted dry with absorbent paper. After weighing the fish (with an electronic balance of 0.01 g precision), Tris-HCl buffer (pH = 8.0) was added at a ratio of 1:5 (g:mL) into a homogenizer for homogenization (performed in an ice bath to maintain enzyme activity). The homogenate was centrifuged at 6000 r/min for 10 min at 4 °C, and the supernatant (enzyme source supernatant) was used for subsequent biochemical determinations.

For protein content quantification, the BCA method was employed. The BCA method relies on the color reaction between proteins and a BCA reagent. Absorbance was measured

at 595 nm and protein concentration was calculated using a pre-established standard curve. For SOD activity measurement, the pyrogallol auto-oxidation method was used, wherein we quantified the inhibition rate of pyrogallol auto-oxidation by SOD, with absorbance recorded at 325 nm to calculate SOD activity in units per mg protein. For the CAT activity assay, the ammonium molybdate colorimetric method was applied. Residual H_2O_2 after CAT catalysis reacts with ammonium molybdate to form a yellow complex, and absorbance at 405 nm was measured to calculate CAT activity as μmol H_2O_2 decomposed per minute per mL of enzyme solution [24,25].

2.3.3. Data Analysis

Prior to statistical analysis, the normality and homogeneity of variance of all experimental data were tested to meet the prerequisites for parametric tests. The average protein content and enzyme activity (SOD, CAT) in each toxicity test group were calculated first. All data were analyzed using SPSS 23.0 statistical software: one-way analysis of variance (ANOVA) was performed, followed by Tukey's post-hoc multiple comparison test [26], to compare the statistical differences in protein content and enzyme activity among multiple concentration groups and different time points, with the statistical significance set at $p < 0.05$ (95% confidence level). The experimental design (5 fish per beaker, four parallels per group) ensured sufficient statistical power to detect potential differences, and repeated verification further confirmed the reliability of the statistical results. Graphs were plotted using Origin2022 software for visualizing the statistical results.

3. Results

3.1. Acute Toxicity of NDMA to Juvenile Crucian Carp

In the 96 h acute toxicity pretest, all juvenile crucian carp in the 40–200 mg/L NDMA concentration groups died within 24 h, while those in the control group and 0.4–1.2 mg/L groups survived for 96 h (Table 1). The LC_{100} and LC_0 of juvenile crucian carp were determined as 40.0 mg/L and 1.2 mg/L, respectively. This rapid and complete mortality at high NDMA concentrations is attributed to severe acute toxic effects, likely causing overwhelming physiological stress and disruption of vital regulatory functions that exceeded the tolerance capacity of the juvenile crucian carp [27].

Table 1. Preliminary test of acute toxic effects of NDMA on crucian carp seedlings.

NDMA Concentration (mg/L)	Test Total (Fish)	24 h Survival Number (Fish)	48 h Survival Number (Fish)	72 h Survival Number (Fish)	96 h Survival Number (Fish)
200	20	0	0	0	0
160	20	0	0	0	0
120	20	0	0	0	0
80	20	0	0	0	0
40	20	0	0	0	0
10	20	20	18	12	12
4.8	20	20	20	18	18
1.2	20	20	20	20	20
0.8	20	20	20	20	20
0.4	20	20	20	20	20
blank control	20	20	20	20	20

Based on the pretest results, eight concentration gradients (1.20, 1.86, 2.88, 4.47, 6.92, 10.72, 16.60, 25.70 mg/L) were designed using the equal logarithmic spacing method, with three parallels per group and one blank control group (Table 2). During the 96 h formal test, no mortality was observed in the control group and 1.20–1.86 mg/L groups; mortality

began to increase rapidly in the 2.88–4.47 mg/L groups after 48 h, and mortality reached 96.67% in the 25.70 mg/L group at 96 h (Figure 2). These mortality data formed the basis for the subsequent LC_{50} calculations.

Table 2. Death of crucian carp seedlings within 96 h.

Experimental Treatment	Concentration (mg/L)	Test Total (Fish)	Number of Death/Fish											
			24 h			48 h			72 h			96 h		
blank control	0	20	0	0	0	0	0	0	0	0	0	0	0	0
NDMA	1.20	20	0	0	0	0	0	0	0	0	0	0	0	0
	1.86	20	0	0	0	0	0	0	0	0	0	0	0	0
	2.88	20	0	0	0	2	3	2	3	2	3	3	3	3
	4.47	20	0	0	0	3	5	4	6	5	4	6	5	5
	6.92	20	0	6	5	6	5	6	7	6	6	7	8	8
	10.72	20	10	9	9	10	9	9	11	11	11	12	12	12
	16.60	20	16	15	15	16	15	15	16	16	16	18	18	18
	25.70	20	18	17	16	19	18	18	19	19	18	20	19	19

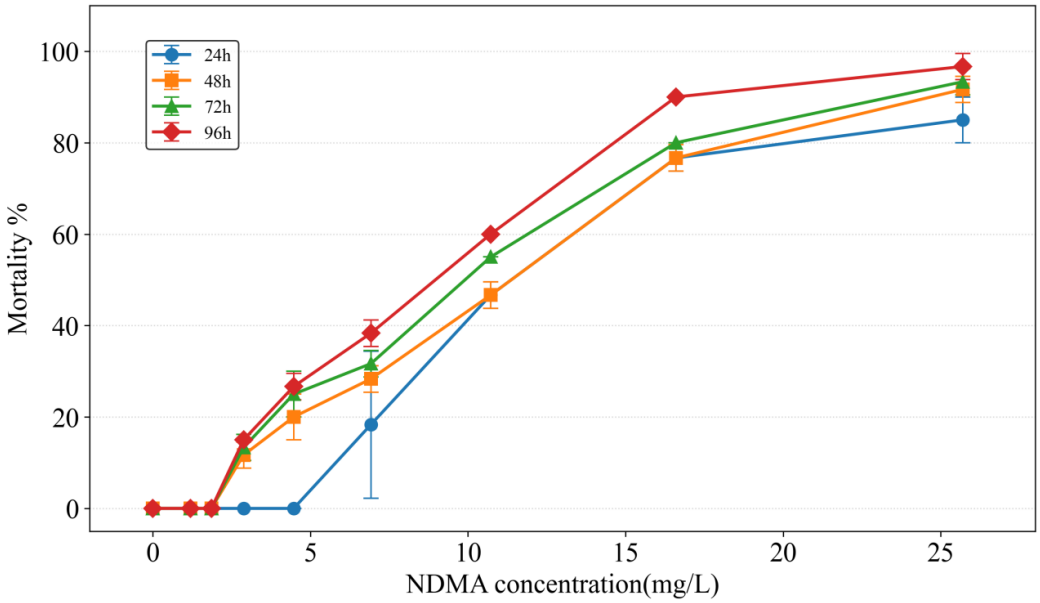


Figure 2. Mortality trend plot of crucian carp hatchlings exposed to NDMA from 24 h to 96 h.

Using linear interpolation and linear regression analysis (Tables 3 and 4), the 24/48/72/96 h LC_{50} values of NDMA on juvenile crucian carp were calculated as 9.83, 9.14, 8.11, and 7.05 mg/L, respectively, with a safe concentration (SC) of 0.705 mg/L. According to the acute toxicity classification standard (Table 5), NDMA is a medium toxic substance to juvenile crucian carp.

The toxicity accumulation coefficient was used to analyze the accumulation and degradation changes of NDMA in juvenile crucian carp. The relevant calculation formulas are as follows:

$$SC = 96\text{h}LC_{50} \times 0.1 \quad (1)$$

$$MAC = \frac{(t_1LC_{50} - t_2LC_{50})}{(t_0LC_{50} - t_mLC_{50})} \times 100\% \quad (2)$$

In the formula, LC_{50} is the half lethal mass concentration (mg/L), SC is the safe mass concentration (mg/L), t_1LC_{50} and t_2LC_{50} are the half lethal mass concentration values (mg/L) for observation periods t_1 and t_2, and t_0LC_{50} and t_mLC_{50} are the half lethal concentration values (mg/L) at the beginning and end of the experiment.

The toxicity accumulation coefficient (MAC) values of 24–48 h, 48–72 h, and 72–96 h were 38.76%, 57.87%, and 59.55%, respectively. The MAC value showed an upward trend overall, but the increase rate slowed down in the 72–96 h period.

During the exposure period, the juvenile crucian carp were visually observed for general behavioral and clinical conditions. No obvious abnormal external signs were observed in the control and low-concentration groups, while fish exposed to higher NDMA concentrations exhibited mild stress-related behavioral changes, such as reduced swimming activity and slower responses to external stimuli.

Table 3. Results of the acute toxicity test of NDMA on crucian carp seedlings.

NDMA	Logarithmic	Mortality Rate (%)			
Concentration (mg/L)	Concentration (mg/L)	24 h	48 h	72 h	96 h
0.00	0.00	0.00	0.00	0.00	0.00
1.20	0.08	0.00	0.00	0.00	0.00
1.86	0.27	0.00	0.00	0.00	0.00
2.88	0.46	0.00	11.67	13.33	15.00
4.47	0.65	0.00	20.00	25.00	26.67
6.92	0.84	18.33	28.33	31.67	38.33
10.72	1.03	46.67	46.67	55.00	60.00
16.60	1.22	76.67	76.67	80.00	90.00
25.70	1.41	85.00	91.67	93.33	96.67

Table 4. Regression equation of toxicity of NDMA to crucian carp seedlings and LC_{50}.

Test Agent	Exposure Time/h	Regression Equation	R^2	LC_{50}
NDMA	24	y = 67.073x − 16.583	0.8068	9.83
	48	y = 65.144x − 12.583	0.9077	9.14
	72	y = 68.308x − 12.087	0.9328	8.11
	96	y = 73.653x − 12.478	0.9403	7.05

Table 5. Classification of acute toxicity of chemical substances.

Acute Toxicity Grade	Highly Toxic	High Toxic	Medium Toxic	Low Toxic
Concentration (mg/L)	<0.1	0.1–1.0	1.0–10.0	>10.0

3.2. Toxic Effects of NDMA on Physiological and Biochemical Indexes of Juvenile Crucian Carp

3.2.1. The Effect of NDMA on Protein in Juvenile Crucian Carp

The test set included five concentration gradients: blank control, safe concentration (0.71 mg/L), 1/8 96 hLC_{50} (1.18 mg/L), 1/4 96 hLC_{50} (1.76 mg/L), and 1/2 96 hLC_{50} (3.68 mg/L). The protein content of the control group was 14.33 mg/g at the initial stage (Figure 3a).

After 1 d of exposure, the protein content of the 0.71, 1.18, 1.76, and 3.68 mg/L groups increased by 7.40%, 16.47%, 36.66%, and 23.49%, respectively, compared with the control group. At 3 d, the increase rates were 7.80%, 19.70%, 33.93%, and 17.20% ($p < 0.05$), with the 0.71 mg/L group showing the slowest growth (15.76 mg/g, $p < 0.05$). At 5 d, the protein content was the lowest in the 0.71 mg/L group (16.30 mg/g) and the highest in the 1.76 mg/L group (19.63 mg/g, $p < 0.05$), with the growth rate of the latter decreasing to 31.22%.

As shown in Figure 3b, the protein content of all experimental groups was higher than that of the control group during the 5 d exposure. The 1.18 mg/L group showed the fastest

increasing trend over time, while the 1.76 and 3.68 mg/L groups showed a decreasing trend from 4 d to 5 d.

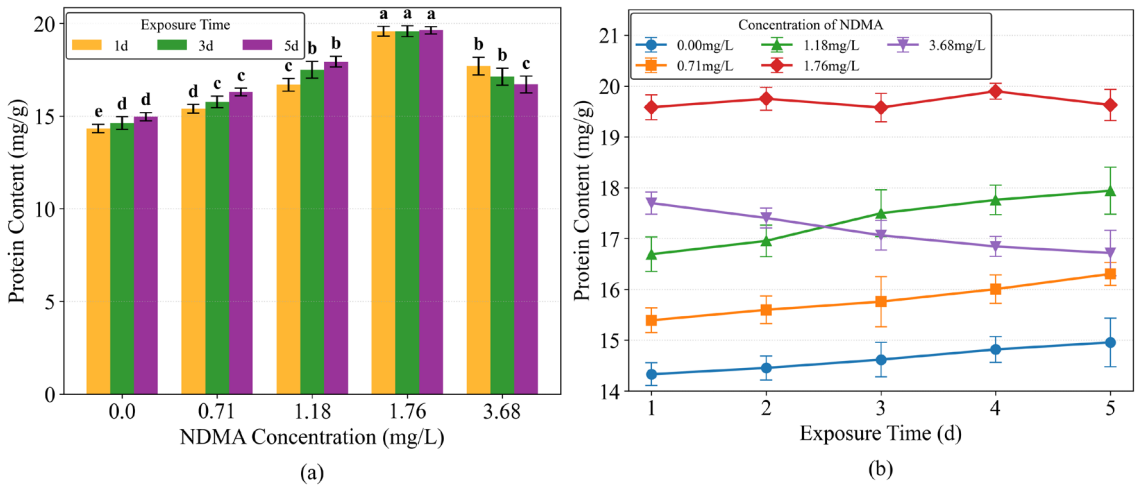


Figure 3. (**a**) Changes of protein content of juvenile crucian carp under stress. (**b**) Protein content in crucian carp hatchlings after 1 to 5 days of NDMA exposure (Different letters represent significant differences, $p < 0.05$).

3.2.2. Effect of NDMA on SOD Enzyme Activity in Juvenile Crucian Carp

After 1 d of exposure, the SOD activity of the 1.18, 1.76, and 3.68 mg/L groups was significantly different from that of the control group ($p < 0.05$; Figure 4a). Compared with the control group, the 0.71, 1.18, and 1.76 mg/L groups increased by 1.70% ($p > 0.05$), 9.49%, and 12.39% ($p < 0.05$), respectively, while the 3.68 mg/L group decreased by 4.51% ($p < 0.05$).

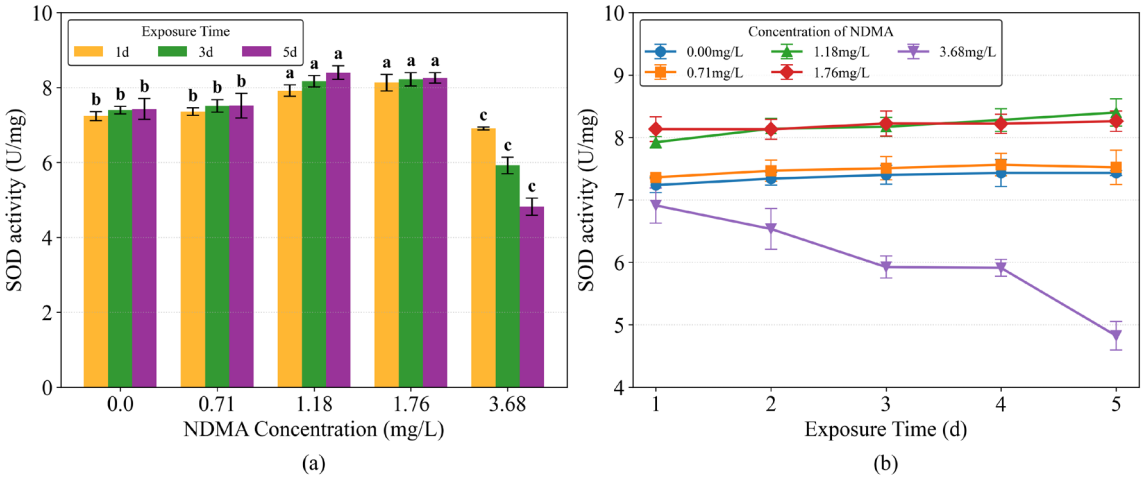


Figure 4. (**a**) Changes of SOD activity of juvenile crucian carp under NDMA stress. (**b**) SOD activity in crucian carp hatchlings after 1 to 5 days of NDMA exposure (Different letters represent significant differences, $p < 0.05$).

At 3 d, the SOD activity of the control group was 7.40 U/mg. The 0.71 mg/L group increased by 1.44% ($p > 0.05$), the 1.18 and 1.76 mg/L groups increased by 10.41% and 11.13% ($p < 0.05$), and the 3.68 mg/L group decreased by 19.96% ($p < 0.05$) to 5.92 U/mg.

At 5 d, the SOD activity of the 3.68 mg/L group was the lowest (4.82 U/mg), decreasing by 35.08% compared with the control group ($p < 0.05$). The 0.71 mg/L group increased by 1.21% ($p > 0.05$), and the 1.18 and 1.76 mg/L groups increased by 13.06% and 11.17% ($p < 0.05$).

As shown in Figure 4b, during the 5 d exposure, the SOD activity of the 1.18 and 1.76 mg/L groups remained significantly higher than that of the control group ($p < 0.05$). In

contrast, the SOD activity of the 3.68 mg/L group was significantly inhibited, showing a gradual decreasing trend with prolonged exposure, and reached the minimum value at 5 d.

3.2.3. Effect of NDMA on CAT Enzyme Activity in Juvenile Crucian Carp

After 1 d of exposure, the CAT activity of the 1.18, 1.76, and 3.68 mg/L groups increased significantly ($p < 0.05$; Figure 5a), with increases of 21.26%, 46.50%, and 13.01%, respectively. The 1.76 mg/L group showed the highest value (356.93 μmol/min·mL).

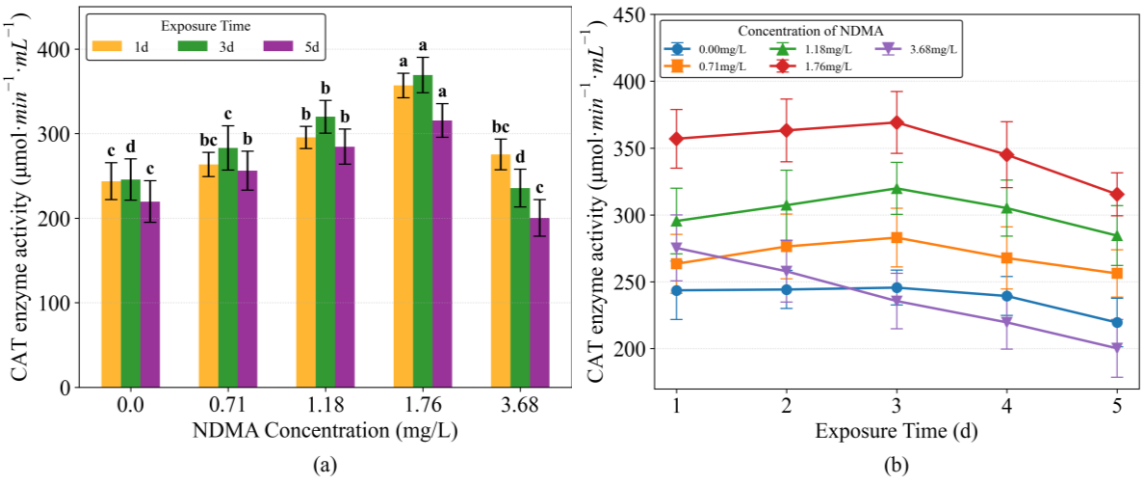


Figure 5. (**a**) Changes of CAT enzyme activity in juvenile crucian carp under NDMA stress. (**b**) CAT enzyme activity in crucian carp hatchlings after 1 to 5 days of NDMA exposure (Different letters represent significant differences, $p < 0.05$).

At 3 d, the CAT activity of the 1.18 and 1.76 mg/L groups increased by 30.21% and 50.28% ($p < 0.05$), while the 3.68 mg/L group decreased to the control level ($p > 0.05$).

At 5 d, the CAT activity of the 1.18 and 1.76 mg/L groups was 284.51 and 315.40 μmol/min·mL ($p < 0.05$), respectively, while the 3.68 mg/L group decreased significantly to 200.27 μmol/min·mL ($p < 0.05$).

As shown in Figure 5b, during the 5 d exposure, the CAT activity of the 1.18 mg/L group remained stably higher than that of the control group. The 1.76 mg/L group showed a trend of increasing first and then decreasing, reaching the peak at 3 d and declining slightly at 5 d. The 3.68 mg/L group showed a continuous decreasing trend with prolonged exposure, and was significantly lower than the control group at 5 d.

3.2.4. Effects of NDMA on Various Physiological and Biochemical Indicators in the Bodies of Juvenile Crucian Carp

The inhibition rate of NDMA on various physiological and biochemical indicators in juvenile crucian carp was calculated according to Formula (3) [28]. When the inhibition rate (IR) is greater than zero, it indicates that the toxin exhibits an inhibitory effect on the organism; when IR is less than zero, it indicates that the toxin has an inducing effect on the organism.

$$IR = (1 - \frac{N}{N_0}) \times 100\% \quad (3)$$

where IR is the inhibition rate; N is the experimental group data value; and N_0 is the blank control group experimental value.

The results showed that protein content showed an induction effect in all concentration groups during the 5 d exposure (Figure 6a–c). SOD activity was induced in the 1.18 and 1.76 mg/L groups and inhibited in the 3.68 mg/L group. CAT activity was mainly induced, with inhibition only observed in the 3.68 mg/L group at 3–5 d.

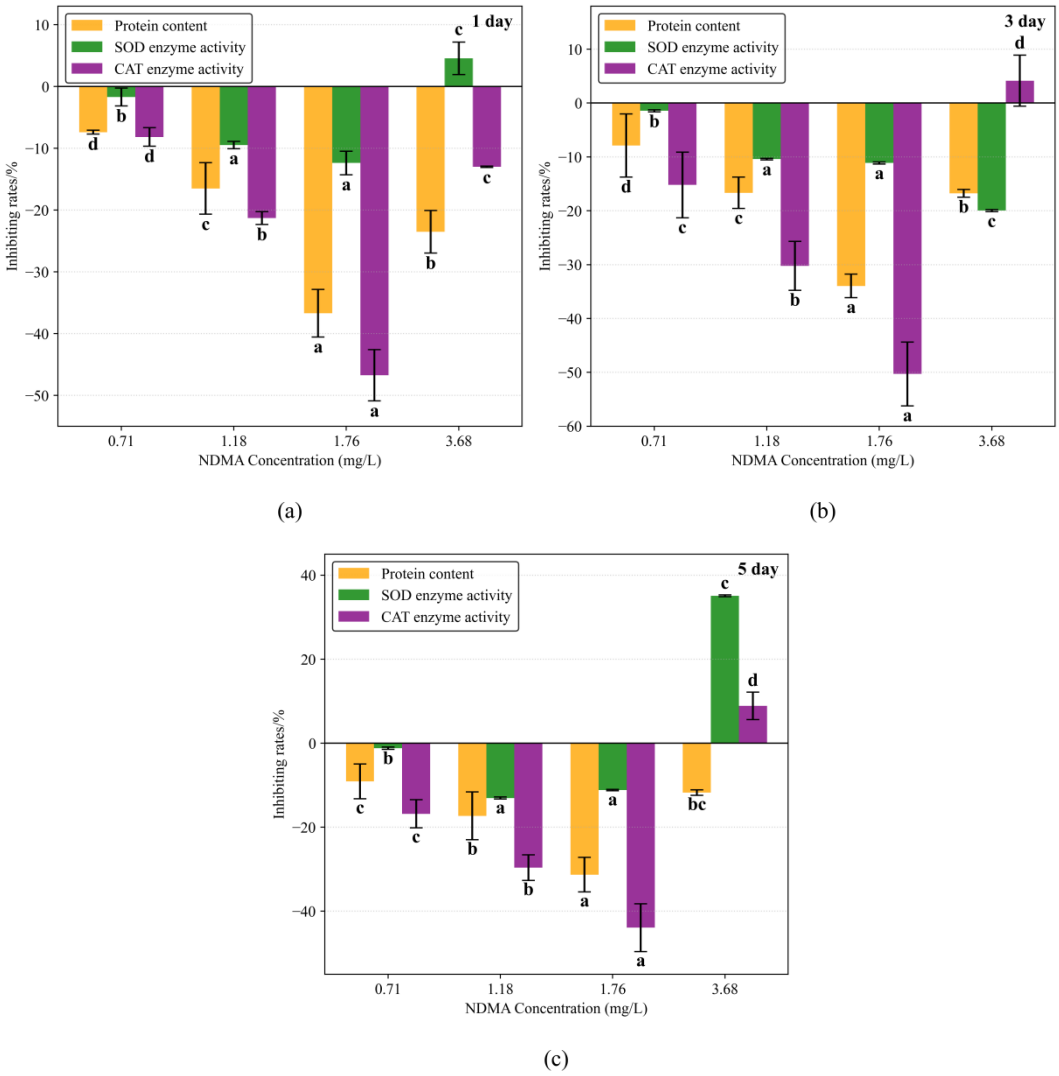


Figure 6. Effects of NDMA on physiological indexes of crucian carp hatchlings. (**a**) Infected 1 day, (**b**) Infected 3 day, (**c**) Infected 5 day. Different letters represent significant differences, $p < 0.05$.

Overall, the inhibition rate of NDMA on the three indicators followed the order: SOD enzyme activity > CAT enzyme activity > protein content.

3.3. Gross Morphological Changes of Juvenile Crucian Carp Exposed to High-Concentration NDMA

After 5 days of exposure, it was observed that compared with the control group (Figure 7a), which exhibited a bright silvery-gray body color, intact scales, transparent and stretched fin rays, and a natural swimming posture, juvenile crucian carp exposed to NDMA showed only mild concentration-dependent gross morphological abnormalities. In the 1.76 mg/L exposure group (Figure 7b,c), most individuals showed no obvious morphological abnormalities, with only a few exhibiting mild changes including slightly dull body color, moderately increased and slightly turbid mucus secretion, and mild congestion at the base of the caudal fin, with no obvious petechiae or postural imbalance. In the 3.68 mg/L high-concentration exposure group (Figure 7d–g), most individuals still showed no severe morphological damage, but a few exhibited significant and typical morphological abnormalities, with an obviously dull and blackened body color, severely increased and turbid mucus secretion, complete loss of scale luster, slight abdominal distension, and unbalanced swimming posture in some individuals, as well as obvious

congestion and redness at the base of the caudal fin, curled and pale fin tips with loss of stretching ability, congestion and redness around the eyes, scattered petechiae on the operculum and mandible, and dull, lusterless eyeballs, indicating tissue hypoxia and oxidative damage.

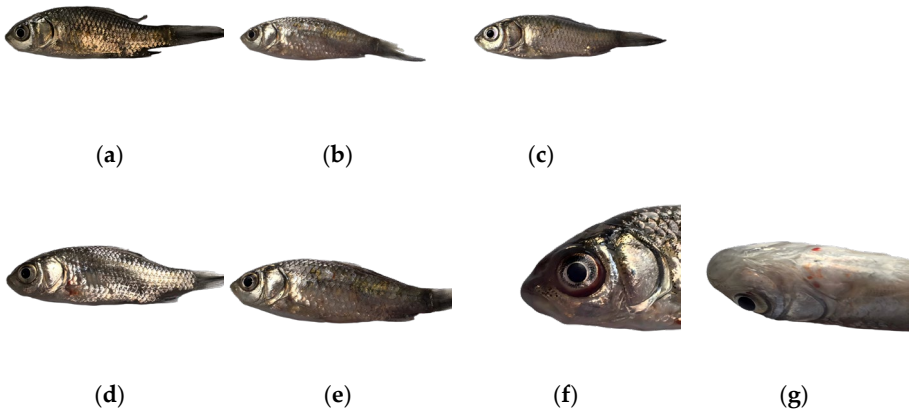


Figure 7. (**a**) Normal gross morphology of juvenile crucian carp in the control group, (**b**,**c**) Gross morphology of juvenile crucian carp in the 1.76 mg/L NDMA exposure group, (**d**–**g**) Gross morphology of juvenile crucian carp in the 3.68 mg/L high-concentration NDMA exposure group.

4. Discussion

The present study systematically evaluated the toxic effects of NDMA on juvenile crucian carp, with comprehensive data providing robust evidence for its ecological risks and toxicological characteristics. A 96 h semi-static acute toxicity test yielded time-dependent half lethal concentrations (LC_{50}) of NDMA: 9.83, 9.149, 8.11, and 7.05 mg/L at 24, 48, 72, and 96 h, respectively, alongside a safe concentration (SC) of 0.705 mg/L. These data confirm that NDMA is categorized as a medium toxic substance to juvenile crucian carp, highlighting its significant acute toxicity at high concentrations. Notably, the gradual decrease in LC_{50} values over time indicates that juvenile crucian carp possess a certain capacity to degrade NDMA, reducing toxin accumulation with prolonged exposure.

The distinct concentration- and time-dependent responses of protein content, SOD, and CAT activities (inhibition rate order: SOD enzyme activity > CAT enzyme activity > protein content) reflect the differential vulnerability of the fish antioxidant defense system to NDMA stress, and these responses can be attributed to the oxidative stress mechanism induced by NDMA with clear molecular and physiological underpinnings. NDMA is metabolically activated in fish hepatocytes primarily via cytochrome P450 (CYP450) enzymes (e.g., CYP2E1)—a key molecular step that generates reactive intermediates (e.g., methyl diazonium ions) and subsequent overproduction of reactive oxygen species (ROS), including superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) [29,30]. At low concentrations (1.18 mg/L and 1.76 mg/L), SOD and CAT activities were significantly induced (up to 1.8-fold and 1.5-fold of the control group, respectively), which is a compensatory physiological response mediated by the Nrf2/Keap1 signaling pathway: ROS activates Nrf2, which translocates to the nucleus and upregulates the transcription of antioxidant enzyme genes (SOD1, CAT) to scavenge excess ROS and maintain cellular redox homeostasis [16,17]. However, high-concentration NDMA (3.68 mg/L) causes persistent ROS accumulation, which overwhelms the antioxidant system and induces oxidative damage to proteins (e.g., carbonylation of enzyme active sites) and lipids (lipid peroxidation), resulting in irreversible inactivation of SOD and CAT—consistent with the "compensation-decompensation" model of oxidative stress [31]. The sustained induction of protein content (up to 1.2-fold of the control) under NDMA stress is a physiological adaptation related to the synthesis of stress-responsive proteins: heat shock proteins (e.g., HSP70) that stabilize

denatured proteins, and metallothioneins that chelate ROS-induced free metals, thereby alleviating cellular damage [18,32].

Molecularly, the higher sensitivity of SOD compared to CAT (inhibition rate: SOD > CAT) can be explained by their distinct roles in ROS scavenging: SOD is the first line of defense against O_2^-, which is highly reactive and rapidly damages nucleic acids and proteins, while CAT primarily decomposes H_2O_2 (a relatively stable ROS) [33]. Thus, persistent O_2^- accumulation under high NDMA exposure directly impairs SOD structure and function, leading to more pronounced inhibition.

Notably, a significant concentration gap exists between the mg/L-level doses in this laboratory acute toxicity test and the ng/L-level NDMA concentrations in natural environments (e.g., groundwater, drinking water). The use of relatively high concentrations adheres to standard aquatic acute toxicity test design principles, intended to rapidly characterize NDMA's dose-response relationship to juvenile crucian carp, identify key toxicity endpoints, and establish toxicity thresholds for subsequent ecological risk assessments. Furthermore, the acute toxicity data from this study (e.g., 96 h LC_{50} = 7.05 mg/L) provides critical quantitative support for deriving NDMA's environmental quality standards and safety thresholds. The data can be integrated with exposure models to scientifically evaluate the long-term ecological risks of low-concentration NDMA exposure in natural environments.

This study has delineated the core acute toxicity parameters and fundamental oxidative stress response profiles of NDMA in juvenile crucian carp, yet it has certain limitations. It did not perform histopathological examinations of key metabolic and respiratory organs such as the gills and liver, as the primary research objective was to delineate core toxicity thresholds and fundamental stress responses. Given that the study prioritized the validation of the primary oxidative stress pathway, the oxidative stress evaluation focused on two pivotal antioxidant enzymes (SOD, CAT) to verify the stress-inducing effects of NDMA; it did not incorporate other oxidative stress markers (e.g., MDA, GPx). This study also only focused on the juvenile stage of crucian carp without investigating the toxic responses of NDMA to adult individuals of this species.

5. Conclusions

This study confirms that NDMA exerts significant acute toxicity and subacute oxidative stress effects on juvenile crucian carp. The 96 h LC_{50} (7.05 mg/L) and safe concentration (0.705 mg/L) provide a reference for ecological risk assessment in freshwater environments. Protein content showed consistent induction, while SOD (the most sensitive) and CAT exhibited "low-concentration induction, high-concentration inhibition" responses, with toxicity mediated by oxidative stress and associated antioxidant-related signaling pathways. SOD and CAT can serve as potential biomarkers for NDMA pollution monitoring, which furnishes practical technical support for the long-term sustainable monitoring and early warning of NDMA contamination in freshwater ecosystems. Future research will supplement histopathological examinations of core organs and expand the oxidative stress indicator system to improve the multi-dimensional verification of NDMA's toxic mechanisms from macroscopic to microscopic levels. Additionally, exploring the long-term effects of NDMA on different life stages of crucian carp and its potential impacts at the population level will further enhance the ecological relevance of the research. These in-depth explorations and ensuing findings will further optimize the scientificity of sustainable management strategies for freshwater environmental pollution. These efforts will not only provide more robust data support for the ecological risk assessment and pollution control of NDMA in freshwater environments but also better safeguard the sustainability of freshwater ecosystems.

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Informed Consent Statement: Not applicable.

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Abbreviations

CAT	Catalase
DMA	Dimethylamine
DOC	Dissolved Organic Compounds
LC_0	0% Lethal Concentration
LC_{50}	50% Lethal Concentration
LC_{100}	100% Lethal Concentration
MAC	Toxicity Accumulation Coefficient
NDMA	N-nitrosodimethylamine
NDEA	N-nitrosodiethylamine
NDPA	N-nitrosodipropylamine
N-DBPs	Nitrogen Disinfection By-Products
ROS	Reactive Oxygen Species
SC	Safe Concentration
SOD	Superoxide Dismutase

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