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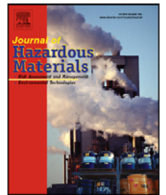

Combined effects of ammonia and microcystin on survival, growth, antioxidant responses, and lipid peroxidation of bighead carp *Hypophthalmythys nobilis* larvae

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ABSTRACT

Hazardous materials, such as ammonia and microcystin, are released into lakes during cyanobacterial bloom degradation and may severely impact aquatic organisms. To assess the combined effects of ammonia and microcystin on survival, growth, and oxidative stress of larval fish, 14-day-old larvae of bighead carp *Hypophthalmythys nobilis* were exposed to solutions with different combined concentrations of ammonia (0, 0.06, 0.264 mg L^{-1}) and microcystin (0, 2, 10, 30 μg L^{-1}) for 10 days. Microcystin significantly decreased body length, while ammonia significantly increased body weight, specific growth rate, and condition factor, but there was no significant interaction between ammonia and microcystin on them. Superoxide dismutase, catalase, and malondialdehyde significantly changed with microcystin concentration, whereas glutathione was not affected by microcystin. Ammonia significantly affected the antioxidant system. There were significant interactions between ammonia and microcystin on superoxide dismutase and malondialdehyde. Our data clearly demonstrate that ammonia and microcystin adversely affect bighead carp larvae.

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1. Introduction

The global intensification of agricultural and industrial activities has enhanced eutrophication in fresh waters, which has led to cyanobacterial blooms increasing in frequency, worldwide [1–3]. Such blooms can result in anoxic conditions and water of poor drinking quality; moreover, they can produce toxins that are released when cells rupture [4–6], such as neurotoxins, hepatotoxins, and endotoxins [7]. Among these toxins, microcystin (MCs), cyclic heptapeptides composed of seven amino acids and of the ≥80 structural variants [8], is the most widely distributed [9] and microcystin-LR (MC-LR) is the most toxic [10].

Concomitant with the release of MCs during blooms degradation, cyanobacteria will produce high ammonia levels, that may persist for days [11]. Such levels may be toxic to a range of organisms, including fish [12]. The combined effects of these two toxins (dissolved ammonia and microcystin-LR) may seriously impact fresh waters [13,14]. Furthermore, there could be synergistic effects of these two compounds.

It is well accepted that MC-LR causes severe liver damage, including massive intraheoatic hemorrhage and liver swelling [15]; also this toxin affects several other processes including cell contractility, membrane transport, and secretion processes, leading to complete disruption of the liver architecture and rapid death [16,17]. Ammonia will affect aquatic organisms in other ways, such as reduced growth [18] and the deterioration of tissue structure, immune function, reproductive capacity, osmoregulatory capability, cell function, and blood chemistry [19,20]. Finally, there is growing evidence that an increase in oxidative damage is involved in the toxicity of microcystins and ammonia [21–25].

Oxidative stress and reactive oxygen species (ROS)-mediated toxicity have long been considered as the mechanisms responsible for environmental condition-induced organ injury in fish [26]. Under normal physiological situations, there is a balance between ROS production and the antioxidant defense system. Cellular oxidative stress occurs when the physiological antioxidant protection does not counteract the elevated ROS levels [27,28]. Antioxidant defenses include a variety of chemical systems, such as glutathione (GSH) and radical scavenging enzymes such as catalase (CAT) and superoxide dismutase (SOD) [29]. These enzymes and non-enzyme play an important role in the antioxidant defense in vertebrates, preventing cells from adverse effects of oxidative stress. When oxidative stress exceeds that of the antioxidant system's removal capability, adverse effects occur, such as an increase

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in lipid hydroperoxides. Malondialdehyde (MDA), an end product of tissue lipid peroxidation, can thus be used as an indicator of oxidative damage [30].

Our target organism to study the effect of these toxins, the bighead carp *Hypophthalmythys nobilis*, is a commonly used aquaculture species [31] and an important component of freshwater ecosystems [32]. Typically, bighead carp larvae are released into lakes, where, as described above, cyanobacterial metabolites and the products of degradation may have deleterious effects. Specially, we investigate a set of key physiological parameters to assess the impact of MCs and ammonia.

It is well known that early life stages are more sensitive to these toxins than adult fish, because of the thinner epithelial layer combined with a relatively larger body surface to volume ratio, high metabolic rate, and limited mobility [33]. In the experiments, therefore, our goal was to use bighead carp larvae as a test organism to determine the responses of growth and antioxidant systems to the toxicity of microcystin in combination with ammonia. Specifically, we tested the following hypotheses: (1) dissolved ammonia and microcystin-LR will have toxic impacts on growth performance of bighead carp larvae; (2) there may be interactive effects between these two toxins that are detrimental to fish larvae growth; and (3) antioxidant parameters will be significantly influenced by ammonia and microcystin, and there may be a significant interaction between ammonia and microcystin on antioxidant parameters. We tested these hypotheses using a range of toxin levels. In fact, our data supported that both ammonia and microcystin are toxic, but rejected most of the hypotheses about interactive effects, interaction only occurred on SOD and MDA levels.

2. Material and methods

2.1. Test organism

Fertilized eggs of bighead carp were obtained from a stock farm (Jiangsu, China), and transferred to the laboratory where they were held in aquaria with 100 L of fresh water and incubated in an aerated tank with 100 L of fresh water at 25 °C. Newly hatched larvae were cultured in aquaria (45 cm × 60 cm × 30 cm). Feeding was administered 3 days after hatching when the vitellus had almost disappeared, and equivalent egg yolk was provided twice everyday. Five days after hatching the larvae were transferred to more aquaria (45 cm × 60 cm × 30 cm) and were fed brine shrimp (*Artemia salina*) nauplii.

2.2. Experimental design

Ammonia test solutions were prepared by dissolving ammonium chloride (NH_4Cl) in de-chlorinated water. NH_3–N concentrations were calculated using the general equation of bases [34]:

$$NH_3 = \frac{[NH_3 + NH_4^+]}{1 + 10^{(pKa-pH)}}$$

The calculation of p*K*a is based on the equation developed by Emerson et al. (1975): $pK_a = 0.09018 + 2729.92/T$, (*T* in °*K*). Based on the toxin levels in the field during degradation of heavy *Microcystis* blooms [6], un-ionized ammonia nitrogen concentrations (NH_3–N) and purified microcystin-LR (MC-LR) concentrations (Express, Beijing, China) were: 0, 0.06, 0.264 mg L^{-1} and 0, 2, 10, 30 μg L^{-1}, respectively. Experiments followed a fully-factorial design; i.e. 12 treatment combinations.

Fourteen-day-old bighead carp larvae [mean ± S.E. 3.5 ± 0.432 mg wet mass (M_w) and 8.986 ± 0.208 mm standard length (L_s)] were randomly assigned to 12, 1000 mL beakers; 50 in each beaker that contained 600 mL water with different concentrations of ammonia and microcystin-LR described above. These treatments were performed in three replicates (i.e. a total of 36 beakers were used), and the experiment lasted 10 days. The experiment was conducted under a 12 L: 12 D photoperiod, at 25 °C and 20 μmol photons $m^{-2} s^{-1}$. To maintain constant NH_3–N and MC-LR concentrations, approximately 30% of the experimental medium was changed every 24 h with fresh solution (preliminary experiment indicated this maintained stable levels) to maintain the initial concentration in a semi-static system. During the experiment, brine shrimp were supplied twice each day.

2.3. Growth

Dead larvae were removed and counted daily. At the end of the trial, ten fish in each tank were randomly selected, anaesthetized, weighed and measured for standard body length (mm). Specific growth rates (SGR) were calculated using the following expression [35], $SGR = [\ln W_f - \ln W_i]/\text{days} \times 100$, where W_i and W_f are initial and final mean wet body weight, respectively. The condition factor (CF) was calculated based on the following formula [36]: $CF = 100WL^{-3}$, where *W* is the weight (g) and *L* is the length (cm) of the fish.

2.4. Antioxidant parameters analysis

To determine the responses in antioxidant parameters of bighead carp larvae, 20 larvae were sampled from each beaker. Antioxidant parameters were measured as whole-body homogenates. Samples were kept on ice during the entire procedure. Whole larvae were rinsed in 0.68% ice-cold physiological saline solution and then dried using filtrating paper [37]. Samples were placed in a Dounce homogenizer with 2 mL 0.68% physiological saline solution and homogenized. Subsequently, whole larvae homogenates were centrifuged at 2000 g for 10 min at 4 °C to eliminate cellular debris and cartilage fragments. The supernatant was removed and used for antioxidant parameters assays. In the experiments, we measured CAT, SOD, GSH and MDA. Protein concentrations were estimated using the Diagnostic Reagent Kit (Coomassie protein assay dye); CAT (U mg^{-1} protein), SOD (U mg^{-1} protein), GSH (mg g^{-1} protein), and MDA (nmol mg^{-1} protein) were determined using the Diagnostic Reagent Kits purchased from Nanjing Jian Cheng Bioengineering Institute (China).

2.5. Statistical analysis

All data are presented as mean ± 1 SE. To test the hypotheses outlined in the Introduction, all parameters were evaluated by two-way (NH_3–N concentration and MC-LR concentration) analysis of variance (ANOVA) followed by Duncan's multiple range test. Statistical significance was established at $P < 0.05$.

3. Results

3.1. Effects of different conditions on survival and growth performance

At the end of the experiment, the survival rates were ~90% in the different treatments, and there was no significant difference among any combinations of NH_3–N and MC-LR (Table 1). The body length was decreased significantly by MC-LR but not by NH_3–N (Table 1, Fig. 1a). Moreover, two-way ANOVA indicated that there was no statistically significant interaction between NH_3–N and MC-LR on body length. NH_3–N significantly promoted body weight, whereas MC-LR did not affect body weight, and no significant interaction was detected between NH_3–N and MC-LR on body weight (Table 1,

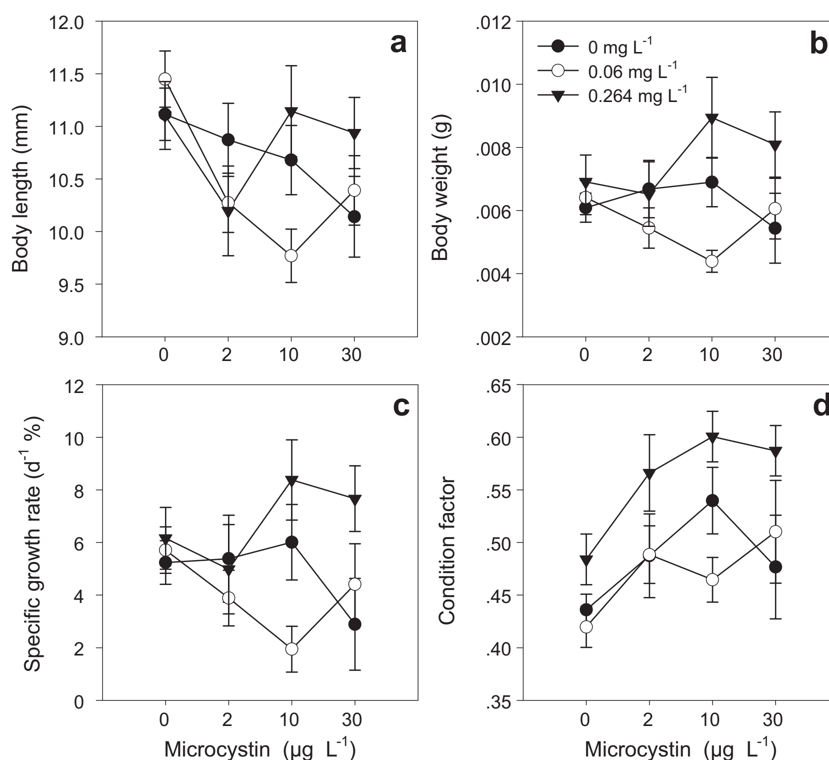

Fig. 1. Changes in body length, body weight, SGR, and CF of bighead carp larvae exposed to different combinations of ammonia and microcystin. Vertical lines represent ±SE.

Table 1
Results of two-way ANOVA on the interaction between microcystin-LR concentration (MC-LR) and un-ionized ammonia concentration (NH_3–N) concentration on survival, growth performance, antioxidant system, and lipid peroxidation of bighead carp larvae.

Parameters	Source of variation	*df*	*F*	*P*
Survival	MC-LR	3	1.936	0.151
	NH_3–N	2	1.000	0.383
	MC-LR × NH_3–N	6	0.361	0.896
Body length	MC-LR	3	3.618	0.016
	NH_3–N	2	1.272	0.285
	MC-LR × NH_3–N	6	2.038	0.067
Body weight	MC-LR	3	0.343	0.795
	NH_3–N	2	6.194	0.003
	MC-LR × NH_3–N	6	1.476	0.193
SGR	MC-LR	3	0.447	0.720
	NH_3–N	2	4.703	0.011
	MC-LR × NH_3–N	6	1.551	0.169
CF	MC-LR	3	4.896	0.003
	NH3–N	2	9.456	<0.001
	MC-LR × NH3–N	6	0.672	0.673
CAT	MC-LR	3	9.706	<0.001
	NH_3–N	2	4.505	0.022
	MC-LR × NH_3–N	6	2.159	0.083
SOD	MC-LR	3	60.514	<0.001
	NH_3–N	2	10.194	<0.001
	MC-LR × NH_3–N	6	4.275	0.005
GSH	MC-LR	3	2.086	0.129
	NH_3–N	2	3.842	0.036
	MC-LR × NH_3–N	6	0.271	0.945
MDA	MC-LR	3	4.504	0.012
	NH_3–N	2	11.748	<0.001
	MC-LR × NH_3–N	6	7.343	<0.001

Fig. 1b). The influences of MC-LR and NH_3–N on SGR were the same as that of body weight (Table 1, Fig. 1c), as SGR is an index calculated from the change of body weight. Both NH_3–N and MC-LR had a significant enhancement on CF, but there was no significant interaction between NH_3–N and MC-LR on CF (Table 1, Fig. 1d).

3.2. Antioxidant enzymes

NH_3–N and MC-LR had a significant effect on CAT; two-way ANOVA indicated that there was no statistically significant interaction between NH_3–N and MC-LR on CAT (Table 1, Fig. 2a). SOD activity also fluctuated significantly with different concentrations of NH_3–N and MC-LR, and there was a statistically significant interaction between NH_3–N and MC-LR on SOD activity (Table 1, Fig. 2b).

3.3. Non-enzymatic antioxidants

MC-LR did not have an effect on the content of GSH, but NH_3–N affected GSH significantly. Two-way ANOVA indicated that there was no statistically significant interaction between MC-LR and NH_3–N on GSH (Table 1, Fig. 2c).

3.4. MDA content

Dose-dependent significant enhancements were observed in MDA content exposure to MC-LR without ammonia (Table 1, Fig. 2d). In contrast, MDA content of the larvae exposed to ammonia decreased with increasing MC-LR concentration. In general, both NH_3–N and MC-LR had a significant effect on MDA (Table 1, Fig. 2d); moreover, two-way ANOVA indicated that there was significant interaction between MC-LR and NH_3–N on MDA (Table 1, Fig. 2d).

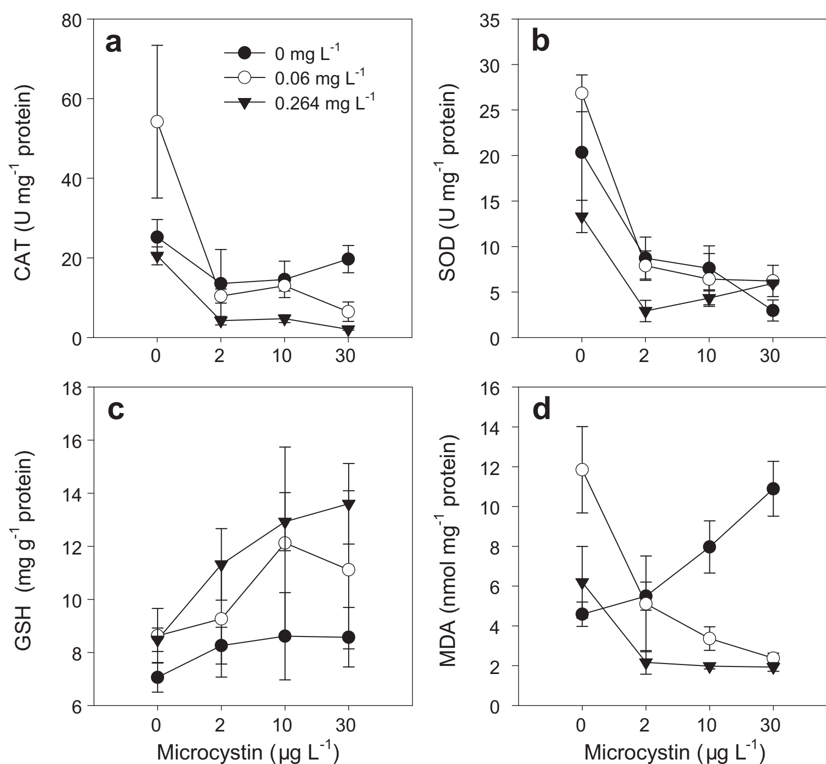

Fig. 2. Changes in antioxidant system and lipid peroxidation of bighead carp larvae exposed to different combinations of ammonia and microcystin. Vertical lines represent ±SE.

4. Discussion

Many authors used cyanobacterial blooms to study the toxic effects of MCs on fish in natural conditions; however the results observed must be attributed not only to MCs, but also to additional compounds present during cyanobacterial degradation [38]. The present study provides clear evidence for the effects of combined factors (MC-LR + NH_3–N) on the fish larvae. Fish larvae are generally considered the most sensitive stage in the life cycle of teleosts, being particularly sensitive to a range of low-level environmental stresses to which larvae may be exposed [39,40]. Therefore, the larvae (14 days) of bighead carp were selected to test the effects of combined factors (MC-LR and NH_3–N). In the present experiment, the whole-body of larvae were homogenized to measure the oxidative stress enzymes because in early life stage, when organogenesis occurs, the toxins may affect the whole organism through action on several organs [39].

4.1. Survival and growth performance

At end of the experiments, the survival rates were around 90% in the different treatments, and all other individuals survived the complete duration of the experiment. Our treatments did not significantly reduce survival of larvae. The present study showed that when exposed only to MC-LR, low doses of MC-LR did not affect the growth of the larvae; however, larval growth (obvious reductions in body length) was inhibited when the larvae were exposed to 30 $\mu g\,L^{-1}$ MC-LR. The findings are consistent with those of Dong et al. [36], who found that low cyanobacteria diet could not inhibit the growth of hybrid sturgeon; however, medium cyanobacteria diet and high cyanobacteria diet could inhibit the growth of hybrid sturgeon [36]. Zhao et al. [41] found that low concentrations of cyanobacteria elevated the SGR of *Oreochromis niloticus*, which may be due to the fact that low dose MC-LR could stimulate fish feeding more food [41]. High dose MC-LR inhibited growth of larvae, this was consistent with previous results in many fish species [42,43]. The inhibition was assumed to result from tissue damage due to the toxin, which was verified by the significant alteration of antioxidant enzyme levels. In our experiments, growth (SGR and body weight) of larvae exposed to water with higher NH_3–N increased significantly compared to those in lower ammonia conditions, as the stimulatory effect of NH_3–N on growth was seen in juvenile salmonids [44]. Madison et al. [45] found that low concentrations of dissolved ammonia could promote growth rate in *Sander vitreus*; Iwata et al. [46] also found high ambient ammonia could promote growth in goby *Mugilogobius abei*. These studies might indicate that the muscle could use external NH_3–N as a supplemental nitrogen source [46]. However, most studies suggested that the NH_3–N could inhibit the growth of fish [47–49]. These inconsistent results from different experiments may suggest that response of fish to ammonia is species-specific under different ammonia concentrations. Moreover, in our study, NH_3–N promoted growth on body weight but not on body length, probably indicating ammonia strengthens allometric growth, which is common in fish [50]. CF, as an integrative indicator, could represent the health condition of larvae. In this study CF changed significantly with various NH_3–N concentrations, and MC-LR also had a significant effect on CF. Decreased CF in fish in response to NH_3–N and MC-LR have been demonstrated in several previous studies [51,52]. In the present experiment, no interactive effect on growth and CF between MC-LR and NH_3–N was observed.

4.2. Antioxidant enzymes

The antioxidant enzymes (SOD and CAT), constitute the major defensive system against ROS [53], and are expected to make a difference in order to eliminate the effect of different concentrations of MC-LR and NH_3–N [22]. In the present study, SOD activity

appeared to be elevated in larvae exposed to the lower concentration of NH_3–N; however, higher concentration of NH_3–N made SOD activity decrease. Hong et al. [54] found that SOD activity of *Eriocheir sinensis* first increased then decreased under increasing NH_3–N concentrations. Similar results were found in submerged macrophyte *Vallisneria natans* [55]. This indicates that a lower dose of NH_3–N could induce organ produce lots of ROS, and the activity of SOD was elevated to deal with excessive ROS [24]; however, under higher doses of NH_3–N, SOD showed lower activity, possibly due to the inability of SOD to overcome extremely high levels of ROS, and excessive ROS could in turn inactivate SOD activity [56]. SOD activity decreased in MC-LR treatments, which was similar to the findings observed in cultured leydig cells exposures to pure MC-LR [57]. Atencio et al. [58] found that tenca (*Tinca tinca*) fed with single doses of *Microcystis* cell (22 μg $fish^{-1}$) also showed the activity of SOD decreased. Exposed to water only with MC-LR, SOD activity gradually decreased with increasing MC-LR concentrations, possibly suggesting that SOD activity decreased due to direct damage of its protein structure by MC-LR and high concentrations of hydrogen peroxide [59]. MC-LR and NH_3–N had a significant interaction on SOD activity and caused severe oxidative stress.

CAT is one of the primary antioxidant enzymes involved in ROS removal. In the present study, a similar tendency as that observed for SOD was found for CAT; the activity of CAT was found to be significantly reduced in MC-LR treatments, suggesting that CAT activity was inhibited by MC-LR. The reduction in CAT activity may be caused by poor efficiency of CAT in removing low ROS levels, or the SOD activity decline made the activity of CAT decrease [60], because the superoxide anion ($O_2^{\bullet -}$) is reduced to H_2O_2 by SOD, and H_2O_2 is converted to water and oxygen by CAT [61]. Modesto and Martinez [59] also reported there is a complex relation between SOD and CAT. Similarly, CAT activity of rats showed a significant decrease, after intraperitoneal injection of pure MC-LR [62]. Li et al. [31] also reported 2 days after intraperitoneal injection of MC extract, the CAT activity in liver of bighead carp decreased significantly. In the treatments only with ammonia, CAT activity increased significantly at 0.06 mg L^{-1} but decreased sharply at 0.264 mg L^{-1}, suggesting CAT can be induced by a slight oxidative stress due to compensatory response but a severe oxidative stress suppresses the activities of this enzyme due to oxidative damage and a loss in compensatory mechanisms [63]. In the present experiment, MC-LR and NH_3–N had no interactive effect on the CAT activity.

4.3. Non-enzymatic antioxidants

As an antioxidant, GSH plays a major role in cellular metabolism and free radical scavenging [64], and it can through the —SH group deal with numerous toxic substances [65]. In this experiment, exposed to single factor NH_3–N, GSH content increased with increasing NH_3–N concentrations. When only considering the influence of MC-LR on GSH content, it seems that GSH content slightly increased with increasing MC-LR concentrations. Increases in GSH level have been observed in response to nonlethal concentrations of inorganic arsenic in acute toxicity experiments [66]. Augmented GSH content was previously reported in *Channa punctatus* after deltamethrin exposure [67]. In the present experiment, increased GSH levels can be considered an adaptive mechanism to combat oxidative stress through an increase in GSH synthesis [63]. Moreover, MC-LR and NH_3–N had no interactive effect on the GSH content.

4.4. Effects of MC-LR and NH_3–N on lipid peroxidation

One of the most damaging effects of these free radicals and their products in cells is the peroxidation of membrane lipids of which MDA is an indicator [30]. MDA is the final product of lipid peroxidation and a sensitive diagnostic index of oxidative injury in cells [68]. Detailed studies have provided evidence that many species exhibit an increased MDA following stress induced by MC-LR [69,70]. In this study, exposed to single factor MC-LR, MDA content increased with increasing MC-LR concentration, which proved that the lipid peroxidation in larvae occurred due to the existence of MC-LR. Zhang et al. [71] have also reported a prominent increase in MDA content of liver in crucian carp *Carassius auratus* injected three times with 150 μg kg^{-1} MC-LR over a 12 h experiment. The increase of MDA indicated that the antioxidant response induced by MC-LR was insufficient to overcome the oxidative stress. It is important to note that higher dose of MC-LR could cause more serious harm for larvae under ammonia conditions. MDA content in exposure to ammonia decreased with increasing MC-LR concentration, probably this was as the result of the decrease in the total polyunsaturated fatty acids which was prone to peroxidative damage [72], demonstrating there was a synergistic effect between ammonia and microcystin on lipid peroxidation.Exposure to the combination of ammonia and MC-LR led to a stress response characterized by significantly decreased SOD activity and MDA content. From the above results it can be concluded that the combination of ammonia and MC-LR may have adverse effects on bighead larvae. Based on our results, during degradation of heavy blooms in lakes, typically, the maximum levels, such as in certain areas of Lake Taihu, are around 10 μg L^{-1} [73], which probably can cause serious harm to fish larvae.

5. Conclusions

It is clear that both MC-LR and NH_3–N induced significant changes in oxidative stress biomarkers at the biochemical level. Microcystin significantly decreased body length, while ammonia significantly increased body weight, specific growth rate, and condition factor, but there was no significant interaction between ammonia and microcystin on them. The study firstly indicated ammonia could aggravate the harmful effect of MC-LR on larvae and cause severe oxidative stress. The more pronounced effects of NH_3–N coupled with MC-LR compared to the MC-LR alone treatment show a synergic effect, this observation is of importance for further assessment studies: since the occurrence of eutrophic lakes is becoming more frequent, cyanobacterial blooms are increasingly causing important fish habitats to contain deleterious levels of MC-LR combined with ammonia. These results provide some evidences on understanding the impact of toxins derived from degradation of cyanobacterial blooms on early stages of fish.

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