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Impacts of Cyanobacterial Toxins from Dau Tieng Reservoir, Vietnam, on the Early Life Stage of Zebrafish

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Abstract: In water bodies, cyanobacteria and their toxins are of serious problem and damage to aquatic organisms including fish. In this study, the zebrafish embryos were exposed to crude extracts of cyanobacterial scum and toxic Microcystis containing 50 and 200 μg L–1 of microcystins (MC), and to crude extract of non-MC producing Arthrospira, until hatching. Then the fish larvae from incubations with 50 μg L^{-1} of MC and *Arthrospira* crude extracts were split into two groups and (1) the first group was raised in control medium; (2) the second group was continuing incubated in the same medium as the embryos were. Another experiment in which fish embryos and larvae were incubated in non-toxic medium was also implemented as control. The results showed that low MC (50 μg L^{-1}) and non-MC crude extracts slightly reduced the hatching rate of the zebrafish embryos. However, high MC (200 μg L^{-1}) crude extracts strongly decreased their hatching rate. Mortality of the zebrafish larvae increased after 11 days of incubation even though they were raised in non-toxic medium. Furthermore, malformation of the fish embryos and larvae was recorded during the experiments.

Keywords: Microcystins, Zebrafish, Hatching Rate, Survival, Malformation

1. Introduction

More than 50% of lakes in Southeast Asian was eutrophic which is favorable condition for cyanobacterial mass development or blooms. Unfortunately, an estimation of 25–75% of these blooms is associated with toxin production (e.g. MC) potently affecting aquatic organisms and human being [1]. Like other aquatic animals, fish are negatively influenced by toxic cyanobacteria and their toxins especially during their bloom decay.

Numerous investigations on bioaccumulation of cyanobacterial toxins (e.g. MC) in fish have been implemented and reported. The toxin MC concentrated mainly in liver but also distributed in gills, muscle, intestine and brain of fish [2].The accumulation of MC in viscera and muscle of fish was reported to reach extremely high concentrations (e.g. up to 39.6 μg MC kg^{-1} of muscle) and went beyond the recommended consumption guideline value of WHO [3].

Microcystin-LR caused a decrease of 40% survival rate of zebrafish larvae at the concentration of 5 and 50 μg L^{-1} and induced 10% body length reduction of the larvae at the higher concentration [4]. Chronic dose of MC-LR affected on the growth of both embryos and larvae of zebrafish as part of energy was demanded for the detoxification processes [5]. MC-LR caused the malformation and lethality of zebrafish embryos and larvae after the embryos were injected with the toxin [6]. Besides, fish respiration and osmotic balance, and

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their behaviors were affected by cyanotoxins [7–9]. Abnormal hatching of loach embryos and juveniles was also induced by MC [10].

After exposure to MC, pathological changes in the liver like loss of architecture, dysfunction, necrosis, apotosis and hemorrhage of hepatocytes, kidney damage and gill degeneration were demonstrated [11–13]. Microinjection showed negative effects on digestive tracts in embryo-larval medaka fish like esophagus, stomach, intestine and associated systems, and 90% survival rate reduction of the embryos after exposure to a sub-lethal dose and higher dose of MC-LR, respectively [14, 15]. MC-LR decreased the glucogen concentration and reduced the protein phosphatase activity in hepatocytes of goldfish [16]. An exposure to 10 µg MC L^{-1} induced the decrease of glutathione and increase of reactive oxygen species activities in carp hepatocytes [17]. Consequently, strong changes of glutathione and reactive oxygen species might lead to oxidant shock in fish hepatocytes.

In general, cyanobacterial toxins especially MC have variety detrimental impacts on fish at different scales, e.g. enzyme activities, cells, organs, stages and species. To our best knowledge, many studies have been conducted to evaluate the effects of cyanobacterial toxins on fish larvae using purified MC but few researches have been done by using cyanobacterial crude extracts. The embryos should be more susceptible to cyanobacterial toxins than adult fish. Therefore, the aim of this study was to investigate the toxicity of crude extracts of cyanobacterial scum containing MC and isolated cyanobacterial strains (one containing and one not containing MC), from Dau Tieng Reservoir, a drinking water supply for million people in Southern Vietnam, to zebrafish at its early life stages.

2. Materials and Methods

Zebrafish, *Danio rerio*, has been raised in artificial medium containing $NaHCO_3$ (75 mg L^{-1}), NaCl (18 mg L^{-1}) and $CaSO_4$ (8.4 mg L^{-1}) [18] in the laboratory of Environmental Toxicology, Institute for Environment and Resources, Vietnam, for two years. Cyanobacterial scum sample (mainly *Microcystis* spp.) and two cyanobacterial strains (*M. aeruginosa* and *Arthrospira massartii*) isolated from Dau Tieng Reservoir were used for experiments. MC analysis by high performance liquid chromatography (HPLC) showed that the scum sample and the *M. aeruginosa* strain contained MC whereas *A. massartii* was negative to MC.

Cyanobacterial crude extracts were prepared according to Pietsch et al. [19] with minor modification. Briefly, dried biomass of scum or GF/C filters containing microbes were homogenized, suspended into reversed osmosis water, sonicated, frozen at –80°C over night and thawed at room temperature. The freeze/thaw cycle was repeated five times. After the last thawing cycle, samples were centrifuged at 4500 rpm, 4°C for 15 min. Supernatants were collected and kept at –80°C prior to the exposures. The crude extracts of cyanobacterial scum and isolate *M. aeruginosa* containing high MC concentrations (6250 µg L^{-1} and 25000 µg L^{-1} MC-LReq, respectively) and crude extract of *A. massartii* at the concentration of 10 g dried weight (DW) L^{-1} were prepared for experiments.

The hatching experiment of zebrafish embryos was conducted according to Oberemm et al. [4]. Briefly, the experiment contained six treatments, 50 fish embryos each (10 embryos per petri disc containing 10 mL of medium, 5 replicates). One treament was raised in non-toxic medium as a control; four other treaments were raised in medium containing 50 and 200 µg MC L^{-1} from cyanobacterial scum or *M. aeruginosa* crude extracts (Ma); and the last one was raised in 10 mg DW L^{-1} of *A. massartii* (Fig. 1), until hatching.

For larvae survival tests, the larvae from exposures to 50 µg MC L^{-1} and crude extract of *A. massartii* were split into 2 groups (24 – 25 in each group). The first group was raised in control medium; whilst the second one was continuing incubated in the same medium as the embryos were. The larvae from control (n = 50) were raised in non-toxic medium (Fig. 1). Every two days, fish media were renewed, and pH and dissolved oxygen of the media were checked (Oxi 197i, Germany). All treatments were run at 25±1 ℃, low-light intensity (~1000 Lux), and light:dark cycle of 12:12 [18] for 11 days. The larvae survival was recorded daily.

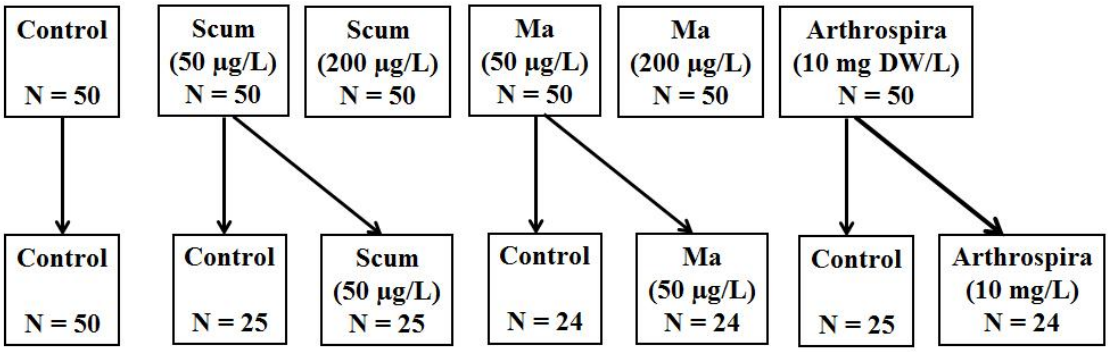

Fig. 1: Experimental set up for hatching rate of zebrafish embryos and survival of larvae

3. Results and Discussion

During exposure period, the dissolved oxygen and pH in the fish media were in the ranges of 5.2–8 mg L^{-1} and 7–8, respectively, which were suitable for the embryo development and larva survival. The results showed that exposures to low MC concentration (50 µg L^{-1}) and non-MC crude extract (*A. massartii*) did not adversely affect the hatching rate of zebrafish embryos. In contract, significant reduction of hatching rate was observed in the treatments with 200 µg MC L^{-1} from both cyanobacterial scum and isolate (*M. aeruginosa*), 40% and 72%, respectively (Fig. 2).

MC induced hyperphosphorylation consequently cytoskeleton impairment [6]. Our results showed that only high MC-containing cyanobacterial crude extract (200 µg MC L^{-1}) decreased the hatching rate of the fish embryos (Fig. 2), which is in agreement with a previous record [12]. However, at 50 µg MC $L^{-1}$from crude extracts and non-MC crude extract did not significantly reduce the hatching rate from our observation which is similar to the results of Oberemm et al. [4]. Properly normal contact to MC would have less potent impacts than the toxin injection into the embryos.

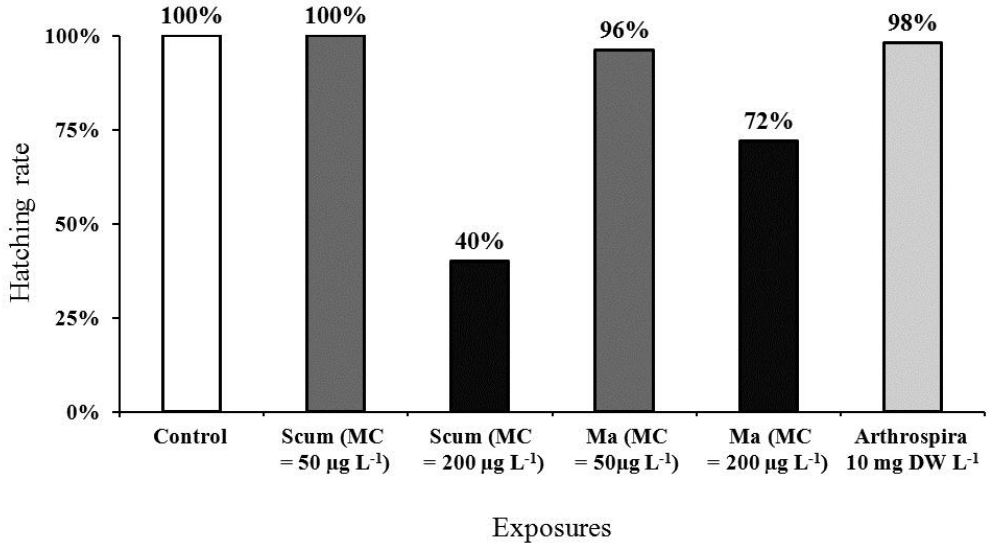

Fig. 2: Hatching rate of zebrafish embryos during incubations. Scum, crude extract from scum; Ma, crude extract of *M. aeruginosa* isolate; *Arthrospira*, crude extract of *A. masarrtii* isolate.

During 11 days of incubation, survival of larvae from control embryos slightly reduced, at 4% of total larvae. However, when the larvae ~~from~~ pre-exposed to non-MC crude extract were raised in control or continuing exposed to the same crude extract, their survival decreased to 68% and 71%, respectively. Seriously, those from embryos pre-exposed to 50 µg MC L^{-1} strongly reduced, at 58–84% of total larvae regardless the larvae were raised in control or toxic medium (Fig. 3). Mortality of larvae was higher when they were continuing exposed to MC medium than to control medium.

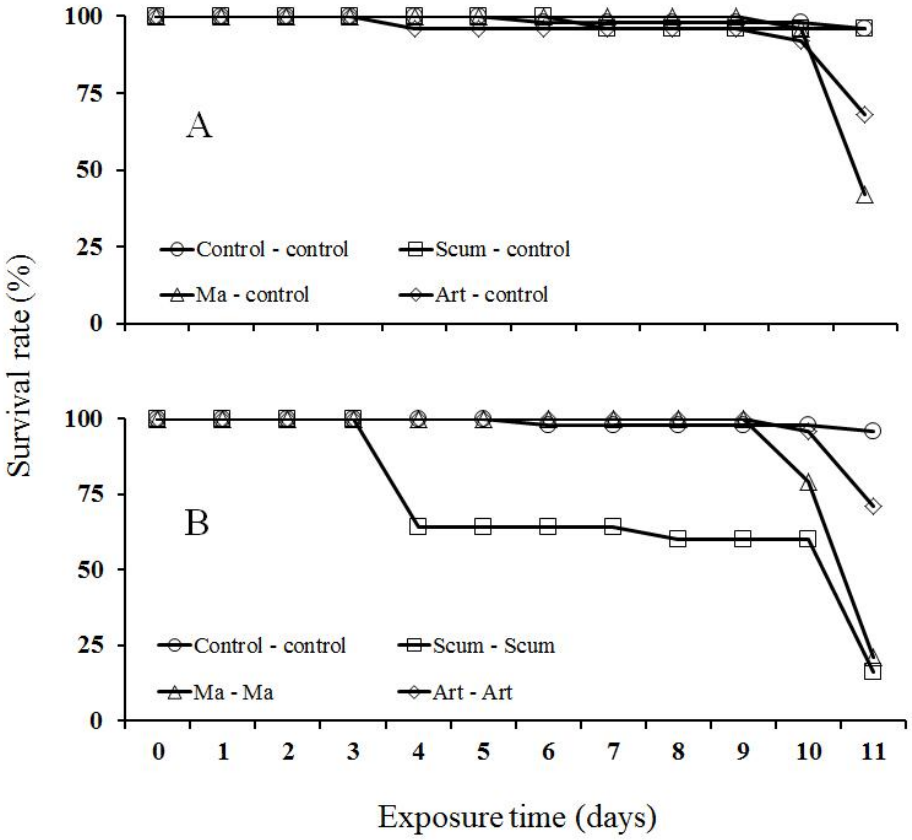

Fig. 3: Survival of zebrafish larvae during the incubations in control medium (A) or cyanobacterial crude extracts (B). Scum, crude extract from scum containing 50 µg MC L^{-1}; Ma, crude extract of *Microcystis* isolate containing 50 µg MC L^{-1}; Art, crude extract of *Arthrospira* at the concentration of 10 mg DW L^{-1}.

Previous studies have proved that MC induced the alteration of biotransformation and antioxidant enzyme activities in fish [5, 17]. Consequently, energy was reduced for normal activities such as growth and survival maintain. Oberemm et al. [4, 20] showed that 5 and 50 µg L^{-1} of MC-LR caused a decrease of 40% survival rate of zebrafish larvae after three weeks of incubation. Our results revealed even more serious effects of crude extracts containing 50 µg MC L^{-1} on the exposed larvae of which their mortality decreased up to 84% after 11 days of exposure (Fig. 3B). This difference could be explained as the used cyanobacterial crude extracts might contain some toxins other than MC. This was supported with the decrease of larvae survival by 30% in exposure to non-MC crude extract (*A. massartii*) though the larvae were raised in medium free crude extract (Fig. 3). Further cyanobacterial bioactive compound (other than MC) analyses from these crude extracts (e.g. LC/MC/MC) are suggested to confirm their toxicity.

All embryos in control hatched and normal larvae were recorded (Fig. 4A, D). However, incubation in medium containing MC or non-MC crude extract, dead and malformation of embryos and larvae were observed (Fig. 4B-C, E-H). Observation on the malformed embryos showed that chorions or egg shells were broken after 1–2 days of exposure (Fig. 4C). Consequently, larvae were released out of chorions when they were not fully developed (Fig. 4F, G). In other cases, malformation and dead of larvae were recorded with some abnormalities of their head and curved body (Fig. 4E, H). Our records are similar to previous reports in which abnormal hatching embryos and juveniles of loach, zebrafish and roach were affected by MC-LR [4, 10, 20]. Our results showed that crude extract of *A. massartii* caused the malformation of embryo but larvae. To our knowledge, this observation together with the mortality increase of exposed larvae is the first record on the adverse effects of non-MC producing *Arthrospira* crude extract on zebrafish. Yet, the mechanisms of the embryos and larvae malformation are un-known and need further investigations.

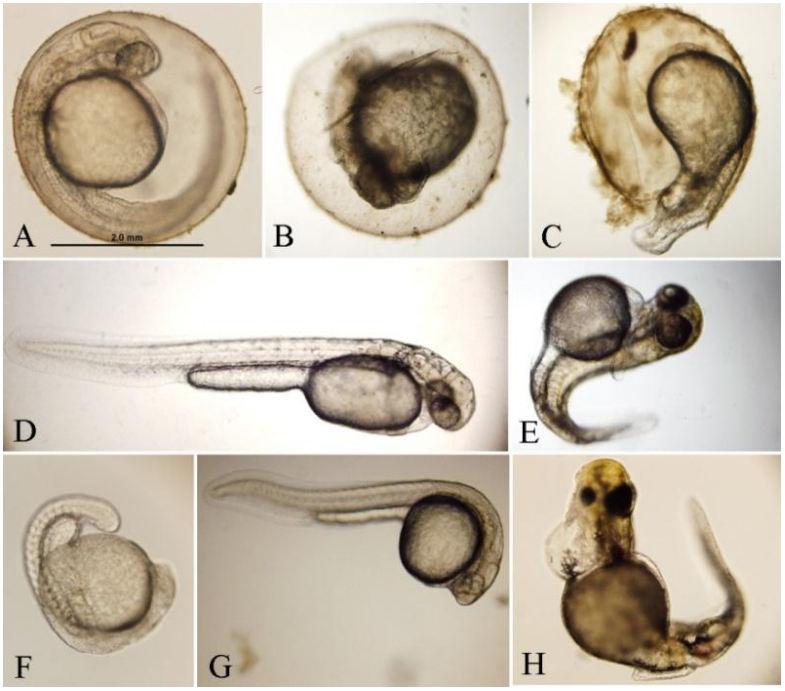

Fig. 4: Malformation of zebra fish embryos and larvae exposed to cyanobacterial toxins. (A), normal embryo; (B-C), malformed embryos; (D), normal larva; (E-H), malformed larvae.

4. Conclusion

Cyanobacterial crude extracts containing MC from Dau Tieng Reservoir, Vietnam, had potently toxic effects on zebrafish. Apparently, high MC concentration (up to 200 µg L^{-1}) strongly inhibited or reduced the hatching rate of zebrafish embryos. Non-MC crude extract and concentration of 50 µg MC L^{-1} did not decrease hatching rate of zebrafish embryos. However, their secondary and long-term effects could be clearly seen in the pre-exposed hatching larvae even if they were raised in toxin-free medium. The detrimental impacts of non-MC producing crude extract from *A. massartii* on zebrafish embryos and larvae, to our best knowledge, are firstly recorded. Further investigations on the malformation mechanisms of zebrafish embryo and larvae exposed to MC are recommended. Also, bioactive compound analyses on cyanobacterial crude extracts from our study (e.g. LC-MS/MS) are suggested to confirm their toxicity.

5. Acknowledgement

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