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Interactive effects of microcystin and ammonia on the reproductive performance and phenotypic traits of the rotifer *Brachionus calyciflorus*

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ABSTRACT

Elevated microcystin-LR (MC-LR) and ammonia (NH_3-N) concentrations co-occur during the degradation of *Microcystis* blooms, and are toxic to aquatic organisms. The freshwater rotifer, *Brachionus calyciflorus*, was exposed to mixtures of MC-LR (0, 10, 30, and 100 $\mu g\ L^{-1}$) and NH_3-N (0, 270, and 540 $\mu g\ L^{-1}$) to assess the combined effects of the two toxicants on reproductive performance and phenotype traits. Single solutions of MC-LR (100 $\mu g\ L^{-1}$) and NH_3-N (540 $\mu g\ L^{-1}$) had negative effects on rotifer reproductive timing and fecundity. Pre- and post-reproductive periods fluctuated with MC-LR and NH_3-N concentrations, while reproductive period and total offspring per female were reduced in mixtures of MC-LR and NH_3-N ($p < 0.05$). Grazing rate of rotifers decreased with grazing time and concentrations of the two toxicants ($p < 0.001$). MC-LR in combination with NH_3-N had negative effects on swimming speed and body length but positively stimulated posterolateral spine development ($p < 0.001$). MC-LR and NH_3-N had synergetic interactive effects on pre-reproductive period, reproductive period, total offspring per female, grazing rate, swimming speed, and body length ($p < 0.05$). In contrast, these effects were antagonistic on post-reproductive period and posterolateral spine length ($p > 0.05$). These results indicate that MC-LR and NH_3-N act synergistically and antagonistically in causing toxicity to *B. calyciflorus* regarding reproductive performance and the formation of defensive phenotypes.

1. Introduction

Harmful cyanobacterium *Microcystis* blooms occur worldwide in eutrophic water bodies and disrupt aquatic ecosystems (Sukenik et al., 2015). The most intriguing characteristic of *Microcystis* is their ability to produce secondary metabolites, cyanotoxins, which are toxic to many aquatic organisms (Leflaive and Ten-Hage, 2007). Microcystins (MCs), cyclic heptapeptides and a main group of cyanotoxins, are mainly retained within the producer-cells during cyanobacterial bloom development (Mowe et al., 2015). MC-LR has a broad range of toxic effects; at a molecular level it mainly inhibits protein phosphatase, which causes intracellular problems in cell growth, differentiation, and osmoregulation (Monserrat et al., 2003; Sun et al., 2012). The main toxic mechanism of MC-LR in vivo is that MC-LR inhibits protein phosphatases type 1 and 2 A (MacKintosh et al., 1990) and induces oxidative stress (Amado and Monserrat, 2010). Furthermore, MC-LR reduces *Brachionus* filtration rate, inhibiting feeding (Liang et al., 2017a). The concentration of dissolved MC-LR in eutrophic waters ranges from trace amounts to 200 $\mu g\ L^{-1}$, but mostly from 0.1 to 10 $\mu g\ L^{-1}$ (Lahti et al., 1997). In a few cases, dissolved MCs levels have reached 1800 $\mu g\ L^{-1}$ or higher in serious eutrophic water bodies during the collapse of highly toxic blooms (Jones and Orr, 1994). The released MCs come into contact with a wide range of aquatic organisms upon which they can have deleterious effects (Chen et al., 2009).

Concomitant with the release of MCs, cyanobacteria produce high levels of ammonia (NH_3-N) during bloom degradation, which is present as ammonium ($NH_4{}^{+}$) and unionized ammonia (NH_3) (Codd et al., 2005). The increasing accumulation level of NH_3-N in water is highly associated with bacterial denitrification and nitrate ammonification (Cheng et al., 2013). Elevated concentrations of NH_3-N reach exceptionally high levels, e.g., 200–3400 $\mu g\ L^{-1}$, in areas of Lake Taihu, China at specific times and with localized peaks of approximately 12,000 $\mu g\ L^{-1}$ during a water crisis (Zhang et al., 2010). Total NH_3-N in localized regions of the Mississippi river can be in the order of approximately 70–4000 $\mu g\ L^{-1}$ (Frazier et al., 1996). High NH_3-N levels are deleterious to zooplankton and result in a wide range of issues in aquatic communities (Arauzo and Valladoli, 2003).

Stressors derived from cyanobacterial blooms often arise simultaneously in waters and pose threats to animals in a combined manner (Cao et al., 2014). Given that MC-LR and NH_3-N co-occur in eutrophic

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waters, one concern includes assessing the extent of synergism and/or additive effects of these two toxicants. The effects of cyanobacteria degradation toxicants on aquatic organisms have attracted the attention of many researchers (Amado and Monserrat, 2010; Ferrão-Filho and Kozlowsky-Suzuki, 2011). Interactions between cyanobacteria degradation toxicants, such as MC-LR and NH_3-N, change the tolerance capacity of zooplankton (Yang et al., 2012). Numerous laboratory experiments showed that a combination of these two derived pollutants of cyanobacterial blooms are toxic to aquatic animals (Cao et al., 2014; Zhu et al., 2015). MC-LR and NH_3-N were reported to have negative effects on the survival and growth of bighead carp *Hypophthalmythys nobilis* larvae (Sun et al., 2012). Yang et al. (2012) confirmed that MC-LR and NH_3-N seriously affect the life-history traits of *Daphnia*, and that the effects of these two toxins are interactive. Dose-dependent reproductive responses of *Daphnia carinata* and *Moina australiensis* to NH_3-N have been well demonstrated (Leung et al., 2011). In contrast with cladocerans and fish, the features of the rotifer life cycle that facilitate their use in ecotoxicological studies are a short life cycle and rapid reproduction (Snell and Janssen, 1995).

Rotifers have multi-organ characters and their sensitivity for chemicals and environmental changes make them useful as in vivo toxicological and lifespan models (Olah et al., 2017). The freshwater rotifer *Brachionus calyciflorus*, as an important member of aquatic food webs, serves as a sentinel species indicating the extent of exposure to toxicants and quantifying toxic effects (Snell and Janssen, 1995). Acute toxicity test with *B. calyciflorus* using cysts to obtain test animals has been accepted as standard method for conducting reproductive toxicity tests (Standard Methods, 1998). Rotifers usually consume algae and cyanobacteria; therefore, grazing of *Microcystis* by *B. calyciflorus* results in acute and chronic toxicity (Liang et al., 2017a). Furthermore, metabolites and degradation products of some cyanobacteria may have deleterious effects on rotifers (Liang et al., 2017b). According to environmental characteristics, most planktonic rotifers exhibit pronounced phenotypic plasticity as means of adaptation (Gilbert and McPeek, 2013; Yin et al., 2017).

Phenotypic plasticity is the ability of a single genotype to produce alternative morphologies, physiological states, or behaviors in response to different environmental regimes (West-Eberhard, 2003). Plastic traits typically comprise the length of spines extending from the anterior, and especially posterior, ends of the body's integumentary skeleton (Gilbert and McPeek, 2013). This plasticity and its control have been most extensively studied in *B. calyciflorus*, where a pair of posterolateral spines may be absent or vary continuously in length from just detectable to half of the body length or more (Schröder and Gilbert, 2009). Behavioral responses, which link the cellular or biochemical level to the organism level, are taken as the first indication of the effects of an environmental perturbation (Chen et al., 2014; Gilbert, 2014). As important behavioral responses, feeding and swimming speed alterations add more sensitivity and ecological relevance to standard toxicity testing and represent transient short-term responses to an external stressor in order to maintain the organism's functions (Garaventa et al., 2010; Liu et al., 2017). There are advantages to using feeding and swimming behaviors as endpoints for assessing aquatic toxicity. Behavioral responses are usually very rapid, occurring in minutes rather than days (Snell and Janssen, 1995). Following a breakout of *Microcystis*, *B. calyciflorus* changes its life strategies (e.g., growth, reproduction, body length, grazing rate, swimming speed, spine length, etc.) in response to the harsher aquatic environment (Liang et al., 2017a). Although several studies on the survival and growth responses of rotifers to MC-LR stress have previously been conducted (Geng and Xie, 2008; Huang et al., 2012), there are few published experimental studies of the combined effects of MC-LR and NH_3-N on reproductive performance and phenotypic traits of *B. calyciflorus*. Thus, it is necessary to explore the interactive effects of MC-LR and NH_3-N on the ecotoxicological parameters and defensive phenotypic traits of rotifers.

The objective of this study was to determine reproductive and phenotypic responses to MC-LR and NH_3-N toxicity using the rotifer *B. calyciflorus* as a test animal. The relative effects of dissolved MC-LR and NH_3-N were evaluated alone and in combination. We hypothesized that most reproductive and phenotypic traits of *B. calyciflorus* can not only be adversely affected by MC-LR and NH_3-N independently, but interactive effects between these two toxicants will occur. This hypothesis was tested by examining a variety of reproductive attributes and phenotypic characteristics over a range of toxin levels. We recognized that MC-LR and NH_3-N have multiple toxic effects, and that these toxicants may act synergistically or additively to cause toxicity.

2. Materials and methods

2.1. Test organism

Brachionus calyciflorus Pallas, 1766, was hatched from resting eggs in artificial freshwater modified Environmental Protection Agency (EPA) medium ($pH = 7.8$, comprising 96 mg of $NaHCO_3$, 60 mg of $CaSO_4 \cdot H_2O$, 123 mg of $MgSO_4$, and 4 mg of KCl in 1 L deionized water at 25 °C) (ASTM, 2001). The rotifer *B. calyciflorus* is an international widely used strain for various types of toxicity assessments (Snell and Janssen, 1995). Experimental results with *B. calyciflorus* provide reference values for aquatic ecotoxicological studies. This rotifer strain was originally collected in Gainesville, Florida, USA in 1983 (Snell et al., 1991) and since then, has been cultured in the laboratory continuously with periodic collection and storage of resting eggs. Feeding was conducted by suspending the green alga *Chlorella pyrenoidosa* (Institute of Hydrobiology, Chinese Academy of Science, Wuhan, China) in the culture medium at 3×10^6 cells mL^{-1}. *B. calyciflorus* strain used in our study was a laboratory clone that has been kept in our laboratory for more than 10 years and has constantly been fed by *C. pyrenoidosa*, which is a palatable food for rotifers in natural freshwater ecosystems. The alga was cultured in 3 L conical flasks containing Bolds Basal Medium. Rotifers and *C. pyrenoidosa* were incubated at 25 ± 1 °C under a 2500-lx light intensity and with a 12 L:12 D photoperiod during the experimental period.

2.2. Experimental design

Many common toxicants in eutrophic freshwaters affect rotifer sensitivity. To evaluate interactive effects of pure MC-LR and NH_3-N on reproductive performance and phenotypic traits of *B. calyciflorus* in serious eutrophic conditions, we purchased MC-LR and NH_3-N from biochemical companies and diluted these compounds to a range of toxin levels which were set based on field observations. MC-LR (purity at least 95% by high-performance liquid chromatography) was purchased from Express Biotechnology Co., Ltd, Beijing, China, diluted with distilled water to a stock solution of 250 μg mL^{-1}, and then diluted to the desired concentrations using EPA medium. NH_3-N test solutions were prepared by dissolving NH_4Cl, which was purchased from Xilong Scientific Co., Ltd, Shantou, China, in EPA medium. NH_3-N concentrations were calculated using the general equation of bases (Emerson et al., 1975):

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

where the calculation of pKa is based on the equation (Emerson et al., 1975): $pKa = 0.09018 + 2729.92/T$, ($T$ is in °K), pH was stable at 7.5. Based on toxin levels in the field during degradation of heavy cyanobacterial blooms, purified MC-LR and NH_3-N concentration treatments were 0, 10, 30, and 100 μg L^{-1} (M_0, M_{10}, M_{30}, and M_{100}), and 0, 270, and 540 μg L^{-1} (N_0, N_{270}, and N_{540}), respectively. The purified MC-LR level was set at 0–100 μg L^{-1}, which is the level at which cyanobacterial blooms collapse in natural waters (Codd et al., 2005) and which was previously shown to have chronic effects on *Daphnia* (Yang et al., 2012; Zhu et al., 2015). The concentrations of NH_3-N solutions were based on field observations (Zhang et al., 2010). Experiments