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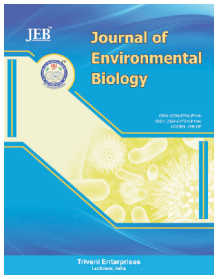

Effect of nitrogen and phosphorus on growth and microcystin production in three *Microcystis* species

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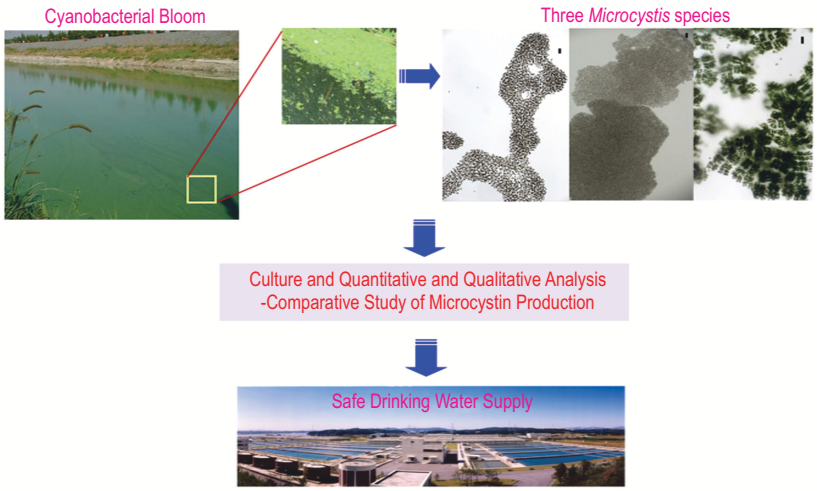
Abstract

Aim : The effects of nitrogen and phosphorus concentration on growth and microcystin production were investigated in three species of bloom-forming *Microcystis* isolated from two South Korean freshwater systems.

Methodology : Three species of cyanobacteria were collected from Yeongchun Dam and Ankei Dam in Kyungpook Province, South Korea. Culture experiments were conducted at 25 ± 1°C under cool white fluorescent light (ca. 50 µmol m^{-2} s^{-1}) in media with different concentrations of nitrogen (0 to 20 mg l^{-1}) and phosphorus (0 to 5 mg l^{-1}). Cell numbers were determined in a hemocytometer for calculation of growth rate. Microcystin was analysed using high pressure liquid chromatography.

Results : The highest growth rate (μ_{max}) and maximal microcystin production occurred at nitrogen concentrations of 10 and 20 mg l^{-1} in all three species. The response to phosphorus concentration was more complex. The highest growth rate (μ_{max}) of *M. aeruginosa*, *M. ichthyoblabe* and *M. viridis* occurred at phosphorus concentrations of 0.5 mg l^{-1}, 0.1 mg l^{-1}, and 3 mg l^{-1}, respectively. *M.aeruginosa* also had maximal microcystin production at 0.5 mg l^{-1} P. In contrast, *M.ichthyoblabe* and *M. viridis* had high microcystin production at 0 mg l^{-1} and at 5 mg l^{-1} P (the highest tested concentration), and low microcystin production at 0.1 mg l^{-1} P. Thus at 0.1 mg l^{-1}, *M.ichthyoblabe* had the highest growth rate but produced least amount of microcystin. The types of microcystins produced varied according to species and nutrient conditions.

Interpretation : Microcystin production and growth in *Microcystis* species isolated from South Korea varied according to species and nutrient conditions. These species responded similarly to different nitrogen concentrations, but differently to different phosphorus concentrations.



Introduction

Cyanobacterial blooms are common in water bodies all over the world, and cyanobacterial toxins are a serious threat to the health of aquatic animals and humans. Microcystins are hepatotoxins produced by certain freshwater cyanobacteria and have well-documented harmful effects in humans (Kuiper-Goodman *et al*., 1999; Falconer, 2001). *Microcystis, Anabaena, Oscillatoria*, *Nostoc and Anabaenopsis* can produce microcystins (Kaebernick and Neilan, 2001) and species of the genus *Microcystis* are the best-known microcystin-producing cyanobacteria (Park *et al*. 1998; Kurmayer *et al*., 2002; Via-Ordorika *et al*., 2004; Ozawa *et al*., 2005; Znachor *et al*., 2006).The genus *Microcystis* consists of 15 species, including *M. aeruginosa*, *M. ichthyoblabe*, *M. flos-aquae*, *M. novacekii*, *M. viridis* and *M. wesenbergii*, all of which have characteristic cell and colony morphologies (Komárek and Anagnostidis, 1999).

Microcystin production is species- and strain-specific (Oh *et al*., 2000; Rohrlack *et al*., 2001; Kurmayer *et al*., 2002; Via-Ordorika *et al*., 2004; Yéprémian *et al*., 2007). Thus, different species and strains produce different type and amount of microcystins, depending on environmental conditions. Studies carried of microcystin production, have mainly focused on *Microcystis aeruginosa* (Codd and Poon, 1998; Watanabe, 1996; Oh *et al*., 2000). However, *M. ichthyoblabe* and *M. viridis* are also responsible for *Microcystis* blooms (Park *et al*., 1998; Sabour *et al*., 2002).

It is necessary to understand the physiological and ecological characteristics of microcystin production by different species of *Microcystis* to better ensure a safe supply of drinking water. Nevertheless, only few studies have compared microcystin production using cultures of different *Microcystis* species and there are no data on the growth of Korean strains of *Microcystis* in culture. In this study, the authors investigated the effects of nitrogen and phosphorus on the growth and microcystin production of three species of bloom-forming *Microcystis* (*M. aeruginosa*, *M. ichthyoblabe* and *M. viridis*) that were isolated from Korean freshwater systems and grown in culture.

Materials and Methods

Strain isolation : Three species of *Microcystis* (*M. aeruginosa* YC, *M. ichthyoblabe* AK and *M. viridis* AK) were isolated from Youngcheon dam and Ankei dam during cyanobacterial blooms. These dams are adjacent and linked by a number of waterways. The species composition of the cyanobacterial communities in two dams were similar at the time of blooms.

Culture conditions : All culture experiments were conducted using CB medium (Watanabe, 1996). Unialgal stock cultures were established and maintained in CB medium (buffered to pH 9.0 with carbon free NaOH) at 25 ± 1°C and a light intensity of approximately 50 μ mol m^{-2} s^{-1} under continuous cool white fluorescent light. For nutrient experiments, clones selected from the stock cultures at the exponential growth phase were first adapted to nutrient-depleted medium (no N or P) for one week, and were then inoculated (initial cell density: ca 5000 cells) into medium with different levels of nitrogen (0, 1, 3, 5, 10, 20 mg l^{-1}) and phosphorus (0, 0.1, 0.5, 1, 3, 5 mg l^{-1}). NO_3^- and PO_4^{3-} were the sources of nitrogen and phosphorus. These cultures were grown for two weeks as described above. Experiments were conducted in triplicate and cell numbers were determined with a hemocytometer following sonication (30 W, 10 sec; Fisher Scientific, USA). Growth rate was calculated as $\mu = (\ln N_2 - \ln N_1) / (t_2 - t_1)$, where N_2 and N_1 are the number of cells during the period of exponential growth at time t_2 and t_1.

Analysis of microcystins : High-pressure liquid chromatography (HPLC) was used to identify and quantify the microcystins. For this analysis, 100 ml of culture was filtered through a 0.45-μm GF/C filter (Whatman, USA) and then lyophilized for 24 hrs in a freeze-drier (Labconco, USA). The lyophilized samples were extracted three times with 50 ml of 5% (v/v) acetic acid for 30 min, while being homogenized with an ultrasonicator (Fisher Scientific, USA).The extract was then centrifuged at 4000 × g for 15 min, and the supernatant was applied to a Sep-Pak C18 cartridge (Waters, USA) that was preactivated by washing with 10 ml of 100% methanol and 10 ml of HPLC-grade distilled water. The cartridge column was washed with 20% methanol and bound microcystins were eluted with 10 ml of 0.1% trifluoroacetic acid in methanol. The eluate was evaporated in a freeze drier (Labconco, USA), and the residue was dissolved in 100% methanol. The solution was then separated by HPLC using an Xterra-C_{18} column (5 μm; 4.6 × 15 cm; Waters) and a mobile phase of 52% methanol and 48% 0.05 M phosphate buffer (pH 3.0) (v/v) at a flow rate of 1 ml min^{-1}. Microcystins were detected by measuring the absorbance at 210–420 nm using a photodiode array (Waters, USA).

Results and Discussion

Fig. 1 shows the effects of nitrogen concentration on the growth rate and microcystin production (mg g^{-1} d.wt.) in *M. aeruginosa* YC, *M. ichthyoblabe* AK and *M. viridis* AK. All three species had maximal growth rates and maximal microcystin production at nitrogen concentrations of 10 to 20 mg l^{-1}.

The specific types of microcystin produced varied according to species and nitrogen concentration. Thus, *M. aeruginosa* YC produced microcystin-YR (MC-YR) when grown in 1-20 mg l^{-1} nitrogen, but also produced microcystin-RR (MC-RR) and microcystin-LR (MC-LR) when grown in 10-20 mg l^{-1} N. M. *ichthyoblabe* AK produced MC-YR when grown in 0-10 mg l^{-1} nitrogen, but produced MC-RR and MC-LR when grown in 20 mg l^{-1} nitrogen *M. viridis* AK produced mostly MC-RR and MC-LR when grown in 3-20 mg l^{-1} nitrogen but produced very little MC-YR at these nitrogen concentrations.

Fig. 2 shows the effects of P concentration on the growth rate and microcystin production of these three *Microcystis*

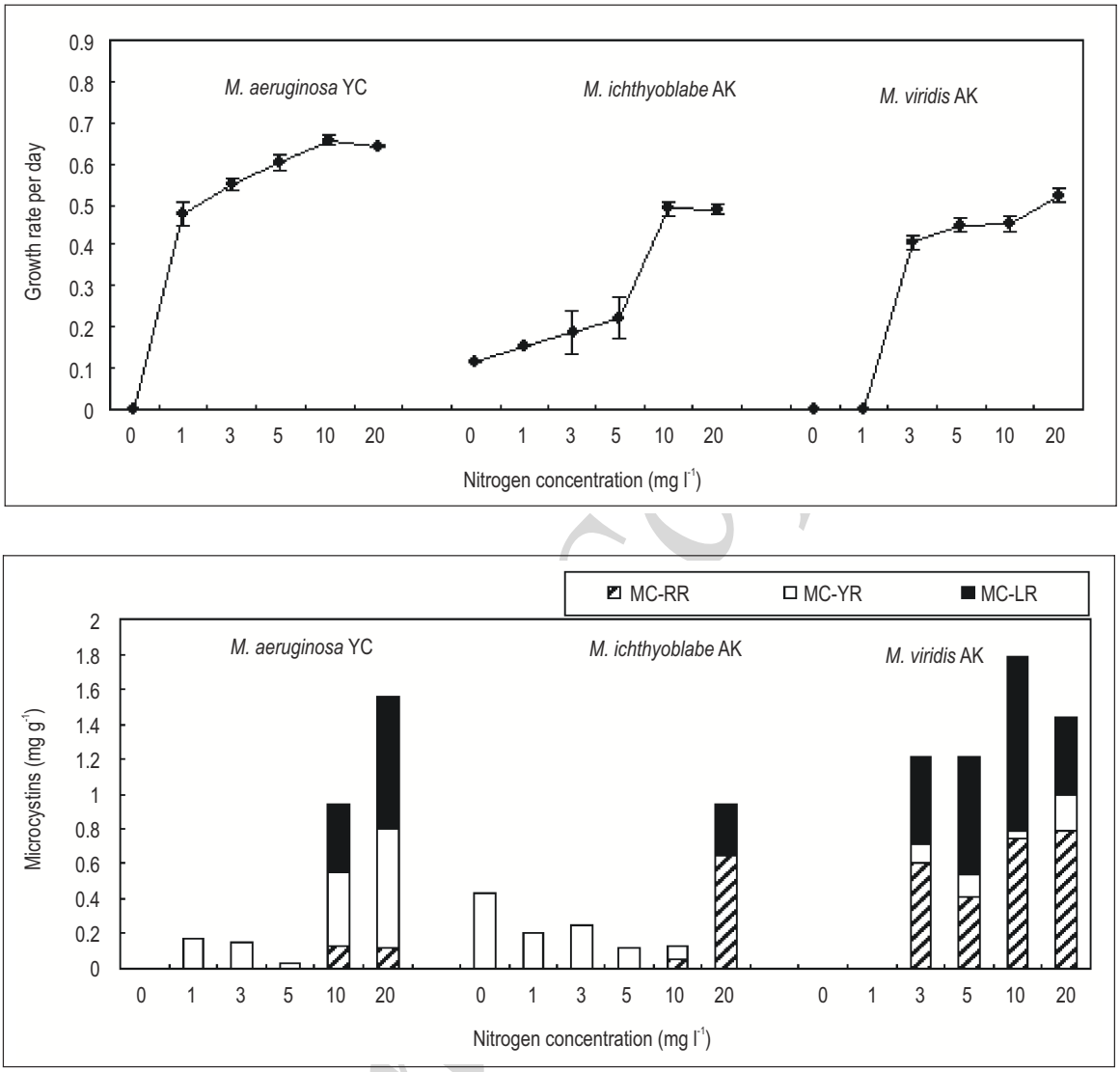


Fig. 1 : Effect of nitrogen on growth rate (top) and microcystin production (bottom) in three *Microcystis* morphospecies

species. *M. aeruginosa* YC had maximal growth rate and microcystin production at 0.5 mg l^{-1}. *M. ichthyoblabe* AK had maximal growth rate at 0.1 mg l^{-1} phosphorus and growth rate decreased gradually as phosphorus concentration increased to 5 mg l^{-1}. In contrast, *M. ichthyoblabe* AK produced maximal microcystin at 0 mg l^{-1} and 5 mg l^{-1} phosphorus, but minimal microcystin at 0.1 mg l^{-1} phosphorus Thus, *M. ichthyoblabe* AK produced more microcystin at phosphorus concentrations that led to lower growth rate.

The growth rate of *M. viridis* AK increased with increasing phoshphorus concentration up to 3 mg l^{-1} and then decreased slightly; its maximum microcystin production was at 0 mg l^{-1} and 5 mg l^{-1} phosphorus (as with *M. ichthyoblabe* AK). Interestingly, although this species did not grow at 0 mg l^{-1} phosphorus, it produced abundant microcystin under this condition. *M. aeruginosa* YC produced all three types of microcystin (MC-RR, MC-YR and MC-LR), but *M. ichthyoblabe* and *M. viridis* only produced two detectable microcystins (MC-RR and MC-LR). *M. aeruginosa* YC produced MC-RR at all tested phosphorus concentrations, and produced the most MC-RR when grown in 0.5 to 5 mg l^{-1} P. *M. ichthyoblabe* AK also produced MC-RR at all tested phosphorus concentrations, but the concentration of MC-LR was only significant at 5 mg l^{-1} P. *M. viridis* AK produced MC-RR at all tested concentrations, but produced nearly similar amount of MC-LR at all tested concentrations.

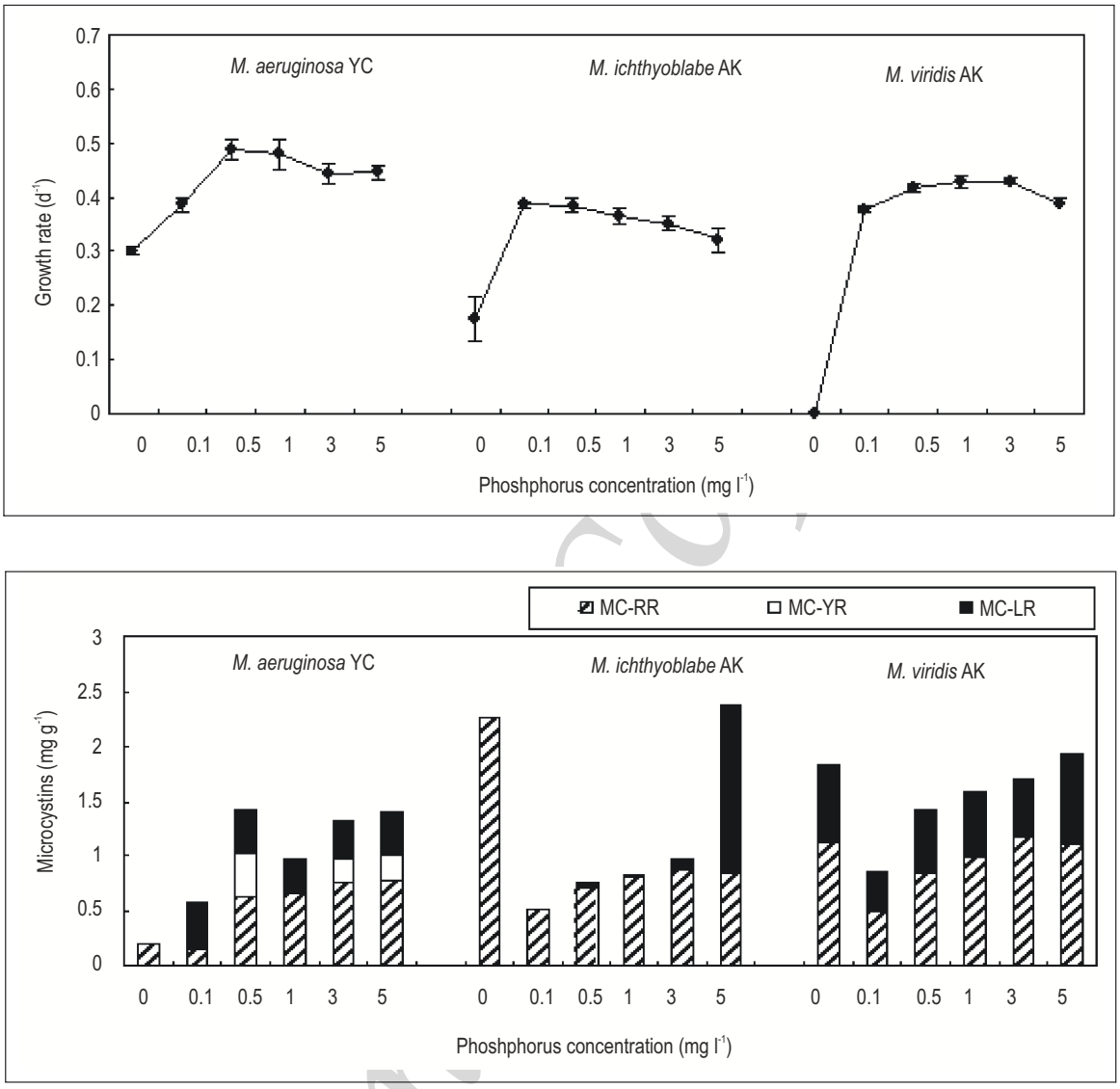


Fig. 2 : Effect of phosphorus on growth rate (top) and microcystin production (bottom) in three *Microcystis* morphospecies

Previous studies have reported that nitrogen concentration affects the production of intracellular microcystins in *Microcystis*, and most of these studies have reported positive correlation between microcystin production and nitrogen concentration (Codd and Poon, 1998; Lee *et al*., 2000), in agreement with the results of this study. In particular, the results on *M. aeruginosa* YC were similar to those of Codd and Bell (1996), who reported that maximal microcystin production occurred at high nitrogen concentrations. Sivonen (1990) also reported that the microcystin levels of a non-N-fixing *Oscillatoria* strain increased as nitrogen concentration increased. However, Rapala *et al*. (1997) found that maximum microcystin production in N-fixing *Anabaena* strain occurred in nitrogen depleted conditions. Non N-fixing cyanobacteria, such as *Microcystis* and certain types of *Oscillatoria*, may require nitrogen for microcystin formation simply because microcystins are nitrogen containing heptapeptide molecules (Sivonen, 1990; Giani *et al*., 2005).

The results suggest that an increased nitrogen concentration in freshwater systems may promote growth and microcystin production by non-N-fixing toxic cyanobacteria. In agreement, previous field studies have reported positive correlation between nitrogen concentration, cyanobacterial biomass and microcystin content (Downing *et al*., 2001; Giani *et al*., 2005). All three species of *Microcystis* produced MC-RR and MC-LR at the highest tested nitrogen concentrations (10-20 mg l^{-1}). These results

are similar to those of Hesse and Kohl (2001), who reported that high concentrations of MC-RR and MC-LR occurred when *Microcystis* grows under nitrogen-rich conditions.

There have been conflicting results on the effect of phosphorus on microcystin production in different cyanobacterial species. Some previous studies of various *Microcystis* strains have reported that phosphorus concentration had little impact on microcystin production (Codd and Poon, 1998; Song *et al*., 1998). Conversely, microcystin production by *Anabaena* (Rapala *et al*., 1997) and *O. agardhii* (Sivonen, 1990) increases with phosphorus concentration under phosphorus-limited conditions. Recently, Oh *et al*. (2000) reported that phosphorus-limiting conditions reduced the growth but increased microcystin content in *M. aeruginosa*. In the present study, it was found that microcystin production by *M. aeruginosa* YC increased with growth rate when the phosphorus concentration was 0-0.5 mg l^{-1}, but additional phosphorus had little or no effect on either parameter. These results are consistent with those of Sivonen (1990), who reported that toxin production by *O. agardhii* increases as phosphorus concentration increases under phosphorus-limited conditions (0.1-0.4 mg l^{-1}). Thus, optimal growth and microcystin production in *M. aeruginosa* YC seem to occur at the same range of phosphorus concentrations. In contrast, maximal microcystin production in *M. ichthyoblabe* AK and *M. viridis* AK occurred at phosphorus concentrations that had the lowest growth rates.

Observations regarding growth and microcystin production in phosphorus depleted conditions could have been affected by incomplete removal of phosphorus that accumulated in cells prior to the experiments. This could explain why microcyst in production in *M. ichthyoblabe* AK and *M. viridis* AK was greatest under conditions of environmental stress (P depletion) and when the growth rate was lowest. However, Hesse and Kohl (2001) reported that production of MC-RR and MC-LR in various strains of *M. aeruginosa* increased in phosphorus-limited conditions. Oh *et al*. (2000) also reported that the ratio of MC-LR to MC-RR in *M. aeruginosa* increased under phosphorus-limited conditions, and that this species produced no detectable MC-YR. The results of *M. aeruginosa* YC indicated that this species produced more MC-RR than other MC variants, MC-RR production increased with phosphorus concentration, and phosphorus had little effect on production of MC-LR and MC-YR. *M. ichthyoblabe* AK produced maximal MC-RR in phosphorus-depleted medium, but produced maximal MC-LR at the highest tested concentration of phosphorus (5 mg l^{-1}). The production of MC-RR in *M. ichthyoblabe* AK was relatively high at all tested phosphorus concentrations.

The effect of different environmental conditions on toxin production differs among cyanobacteria species. *Oscillatoria agardhii* produces more toxins under conditions that support optimal growth (Sivonen, 1990), but the conditions that support optimal growth in *M. aeruginosa* and *M. viridis* do not support optimal toxin production (Watanabe and Oishi, 1985; Song *et al*., 1998). As described above, we found significant species-specific responses for microcystin production under different phosphorus concentrations, but not for different nitrogen concentrations. The findings of this study are thus in agreement with the previous studies (Park *et al*., 1998; Ozawa *et al*., 2005), which reported that microcystin production varied according to the composition of *Microcystis* species where cyanobacterial blooms occur. In addition, differences in microcystin production among species depend on physiological and ecological responses to multiple environmental factors (Rohrlack *et al*., 2001; Yéprémian *et al*., 2007).

In conclusion, the results of the present study show that nutrient levels influence growth and microcystin production in three bloom-forming *Microcystis* species native to Korea, and that nutrient levels have different effects on different species. Further, qualitative and quantitative investigations of cyanobacteria species are needed to improve the management of drinking water supplies that are affected by toxic cyanobacteria, such as *Microcystis*.

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