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Overvalued allelopathy and overlooked effects of humic acid-like substances on *Microcystis aeruginosa* and *Scenedesmus obliquus* competition

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ABSTRACT

To form *Microcystis* blooms, *Microcystis* must be competitively dominant to other phytoplankton species to produce enough biomass. The aim of this study was to determine the competition mechanisms between *Microcystis aeruginosa* and *Scenedesmus obliquus*. *M. aeruginosa* and *S. obliquus* were separately cultured in the filtrate of mono and mixed cultures of *M. aeruginosa* and *S. obliquus* with varying treatments concerning N, P and iron availability. The inhibition rate for *M. aeruginosa* was 20–31% when cultured in mono and mixed culture filtrates enriched with N and P, but this rate was reduced to −15 to 19% when cultured in filtrates enriched with N, P and iron. The inhibition rate for *M. aeruginosa* decreased from 80 to 100% to 11–39% in dialytic filtrates. However, there were no differences in inhibition rate for *S. obliquus,* regardless of filtrate or dialytic filtrate treatments. The potential allelochemical, 2-butyl-octanol (0.144 mg L^{-1}), was found to have little inhibitory effect to *M. aeruginosa* or *S. obliquus*. Thus, previously reported allelopathy to *Microcystis* would be overestimated. We also report a new mechanism in phytoplankton competition in this study. The phytoplankton secreted humic acid-like substances that can reduce the bioavailability of iron, resulting in the inhibition of other phytoplankton.

1. Introduction

Cyanobacterial blooms are occurring more frequently worldwide with increasing global temperature and eutrophication of freshwaters, causing various ecological and environmental problems such as massive fish kills, deteriorated water quality and compromising water supplies (Anderson et al., 2002). *Microcystis* is one of the most widely distributed bloom-forming cyanobacterial species (Harke et al., 2016; Sant'Anna et al., 2008) that produces microcystins which seriously threaten human health (Bell and Codd, 1994; Carmichael, 2001; Falconer et al., 1983; Rao et al., 2002). The proportion of *Microcystis* biomass during blooms has often accounted for more than 95% of the phytoplankton community (Chen et al., 2003). Therefore, *Microcystis* must outcompete other phytoplankton species to produce enough biomass for a bloom.

Organisms, including cyanobacteria and algae, compete against others via two strategies. The first is resource competition, occurring when a specific resource (e.g. nitrogen, phosphorus, iron) is limited. Under this condition, the organism with a lower resource demand may be more successful than other organisms, thereby gradually becoming dominant. The second strategy is interference competition, where one organism inhibits the growth of other organisms directly or indirectly via secretion of chemicals, cell-cell interaction or other biological behaviors. Resource competition has been verified in many nutrient-poor lakes (Holm and Armstrong, 1981; Nagai et al., 2007). However, nutrient concentration in many eutrophic lakes and reservoirs are not usually limited. Because of this, the contribution of allelopathy to the competition among phytoplankton would be higher than resource competition in eutrophic lakes. Moreover, artificially changing nutrient concentration over a short period in lakes and reservoirs is hard to achieve (Le et al., 2010). Therefore, it is feasible to study specific allelochemicals, as well as mechanisms, and artificially synthesize or extract them to inhibit *Microcystis* growth.

Several previous studies focused on allelopathy in phytoplankton competition. Bittencourt-Oliveira et al. (2015) demonstrated that the culture filtrate of *Scenedesmus acuminatus* and *Monoraphidium convolutum* significantly inhibited the growth of *M. aeruginosa* and *M. panniformis*. Zhang et al. (2013) found that the filtrate of *Quadrigula chodatii* inhibited the growth of *M. aeruginosa*. They analyzed the

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filtrate using gas chromatography mass spectrometry (GC–MS), and suggested that dibutyl phthalate and beta-sitosterol may be the allelopathy substances inhibiting growth of *M. aeruginosa*. Similarly, Zhai et al. (2013) reported that D-limonene and 1-chlorine heptacosane, detected from the filtrates of *M. aeruginosa* mono culture and the mixed culture of *M. aeruginosa* and *M. flos-aquae*, may be the allelochemicals inhibiting growth of *M. flos-aquae*. However, all of the above studies only detected possible allelochemicals, but did not verify their inhibitory effect on *Microcystis* growth.

In recent years, fluorescence analysis showed that some phytoplankton, including *Microcystis,* were able to secrete humic acid-like substances (Imai et al., 1999; Sun et al., 2005; Xu et al., 2013). These substances are likely to combine with iron, resulting in reduced iron bioavailability, and thereby inhibiting growth of some other phytoplankton. In addition, various phytoplankton species have different iron utilization abilities (Neilands, 1981; Nagai et al., 2006). Thus, the secreted humic acid-like substances would alter the competition relationship among phytoplankton by reducing iron bioavailability.

The main purpose of this study was to verify the inhibitory effects of allelochemicals on phytoplankton growth, and assess the influence of humic acid-like substances secreted by phytoplankton during competition. If humic acid-like substances influence phytoplankton competition, then previous research regarding inhibition by different phytoplankton culture filtrates may neglect the process and overestimate functions of some allelochemicals. *M. aeruginosa* and *S. obliquus* were chosen as the model strains; *M. aeruginosa* commonly forms harmful blooms (Medrano et al., 2013), and *S. obliquus* has been prevalent in hypertrophic lakes (Trainor, 1998).

2. Materials and methods

2.1. Organisms

Unicellular strains of *M. aeruginosa* (FACHB-469) and *S. obliquus* (FACHB-416) were purchased from the Freshwater Algae Culture Collection, Institution of Hydrobiology of Wuhan Province, Chinese Academy of Science. The two strains were axenically cultured in BG-11 medium for three months prior to experiments.

2.2. Experimental design

M. aeruginosa and *S. obliquus* were mono and mixed cultured in BG-11 culture medium to identify their competitive relationship under standard conditions. Then, both of them were separately mono cultured in the filtrate of both the mono and mixed culture mediums. The filtrate was made with varying treatments (Fig. 1), including: Treatment A, nitrogen (N) and phosphorus (P) concentrations were adjusted to the level of BG-11 medium, Treatment B, N, P and iron concentrations were adjusted to the level of BG-11 medium, Treatment C, the filtrate was first dialyzed and then N and P concentrations were adjusted to the level of BG-11 medium, and Treatment D, the filtrate was first dialyzed and then N, P and iron concentrations were adjusted to the level of BG-11 medium. Each treatment was carried out in triplicate. The concentration of N, P and iron in all the treatments were referring to BG-11 medium because a standard culture with BG-11 medium was set as a control for each treatment. Hence, Treatment B and D was carried out to investigate the effects of all the dissolved organic matters and macromolecular dissolved organic matters (> 1000 Da) in the culture filtrates, respectively, on growth of both *Microcystis and Scenedesmus*. Treatment A and C was carried out to eliminate the effects of iron in Treatment B and D, respectively.

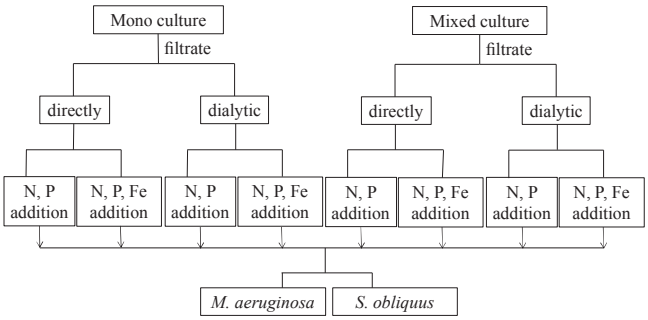

Fig. 1. The filtrate treatments of mono and mixed cultures of *Microcystis aeruginosa* and *Scenedesmus obliquus.*

The composition of metabolites in the filtrate was also analyzed using GC–MS to deduce potential allelochemicals. The effects of these chemicals on algal growth were analyzed to confirm the allelopathy via a 10-day toxicity test, following methods by Wu et al. (2017a, 2017b). In the current study, 2-butyl-octanol may potentially be allelochemical inhibiting the growth of *M. aeruginosa* and *S. obliquus* because it was only found in the mixed culture. *M. aeruginosa* and *S. obliquus* was batch cultured in BG-11 medium (120 mL) in a 250 mL conical with varying concentrations of 2-butyl-octanol. The guarantee reagent 2-butyl-octanol ($C_{12}H_{26}O$), purchased from Aladdin Chemical Reagent Co., Ltd., was used in the toxicity test. The concentrations of 2-butyl-octanol were adjusted to 0, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 mg L^{-1}.

2.3. Culture conditions

M. aeruginosa and *S. obliquus* were cultured in a 250 mL conical flask with 120 mL of BG-11 medium at 25 °C under a 12:12 h light–dark cycle. The initial cell density of *Microcystis* and *Scenedesmus* was 30×10^4 cells mL^{-1} and 5×10^4 cells mL^{-1} because *Scenedesmus* is at least 6 times larger in cell volume than *Microcystis* (Zhu et al., 2016). Light intensity was 50 μmol photons m^{-2} s^{-1}. The cultures were shaken three times every day to prevent cells from adhering to the inner walls of the flasks.

2.4. Filtrate preparation

At the end of the experiment (10 days after incubation), the culture was centrifuged at 10,000 × g for 6 min, and the supernate was filtered using a 0.45 μm membrane. Half of the filtrate was directly used for the second round culture experiment, after adjusting concentrations of N, P and iron to the level of BG-11 medium. The other half was dialyzed via a dialysis bag (Boyuan Biotechnology Company) with 1000 Da. The dialysate, after adjustment of N, P and iron concentrations to the level of BG-11 medium, was used for the second round culture experiment.

2.5. Cell counts

Cell density of both *M. aeruginosa* and *S. obliquus* in mono and mixed cultures was counted under an optical microscope (PH100-2A41L-EP, Phenix) at 400x magnification. To make the measurement more quickly, convenient and consistent, the cell density of the mono culture in the second round was measured using a spectrophotometer (UV-1780, Shimadzu, Japan), based on the relationship between cell density and absorbance at 668 and 680 nm for *Scenedesmus* and *Microcystis*, respectively.

2.6. Chemical analysis

Total dissolved nitrogen (TDN), total dissolved phosphorus (TDP) and total dissolved organic carbon (DOC) concentrations in the filtrate of the first round were analyzed. TDN and TDP concentrations were measured using colorimetry, after digestion with $K_2S_2O_8$ + NaOH (Ebina et al., 1983). DOC concentration was analyzed using a total organic carbon analyzer (TOC-CPN, Shimadzu, Japan). The concentration of iron in the filtrate was analyzed using inductively coupled plasma optical emission spectroscopy (ICP-OES, Perkin Elmer, Optima-