Harmful Algae 61 (2017) 56–62

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Allelopathic effects of *Microcystis aeruginosa* on green algae and a diatom: Evidence from exudates addition and co-culturing

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ARTICLE INFO

Article history:
Received 28 April 2016
Received in revised form 1 November 2016
Accepted 11 November 2016
Available online 1 December 2016

Keywords:
Allelopathy
Cyanobacteria
Eutrophication
Phytoplankton competition

ABSTRACT

Allelopathic interactions among phytoplankton species are regarded as one of the important factors contributing to phytoplankton species competition and succession. The role and extent of allelopathic effects of blooming freshwater cyanobacteria on other phytoplankton species in eutrophied waters, however, are still unknown. We examined the allelopathic effect of *Microcystis aeruginosa* on two common green algae (*Scenedesmus quadricauda*, *Chlorella pyrenoidosa*) and a diatom (*Cyclotella meneghiniana*) by adding exudates from different growth phases and in co-culture tests. Exudates of *M. aeruginosa* from the exponential growth phase and the stationary phase significantly inhibited the growth of *S. quadricauda*, *C. pyrenoidosa and C. meneghiniana*, whereas those from the decline phase increased their growth. The presence of *M. aeruginosa* extremely inhibited the growth of all tested species in co-cultures within 24 h. Our results indicate that under the tested environmental conditions (25 °C, light 80 μmol quanta m^{-2} s^{-1}, manual shaking twice a day), allelopathic effects of *M. aeruginosa* on other phytoplankton species can significantly contribute to their competitive success.

1. Introduction

Toxic cyanobacterial blooms have been a world-wide challenge for decades, and may be facilitated by global warming (Ma et al., 2015; Visser et al., 2016). Bloom-forming species of cyanobacteria in eutrophic freshwater bodies often become problematic due to the release of a variety of toxic secondary metabolites that are not only a health risk for humans (Codd et al., 2005; Lévesque et al., 2016; Testai et al., 2016) but also affect other organisms of different trophic levels: higher plants (Sharma, 1985; Florence and Deborah, 1986; Valdor and Aboal, 2007; Zheng et al., 2013; Corbel et al., 2014) and animals (Codd, 1995; Wu et al., 2012; Hamilton et al., 2014), but also bacteria (Pushparaj et al., 1999; Issa, 1999; Valdor and Aboal, 2007; Martins et al., 2008; Bhattacharyya et al., 2013) and other phytoplankton (Keating, 1978; Suikkanen et al., 2004; Żak et al., 2012; Żak and Kosakowska, 2014).

Both, interspecies competition between cyanobacteria and other algae (Keating, 1978; Suikkanen et al., 2004; Zhang et al., 2013; Bittencourt-Oliveira et al., 2015; Ma et al., 2015) and intraspecies competition within cyanobacteria (Keating, 1977; Pushparaj et al., 1999; Li and Li, 2012; Shao et al., 2013; Rzymski et al., 2014) have been confirmed. Environmental factors such as temperature, light and nutrients were found playing important roles in phytoplankton competition (Marinho et al., 2013; Lei et al., 2015), while some studies have focussed on the influence of allelopathy for species competition or succession (Keating, 1977, 1978; Codd, 1995; Singh et al., 2001; Suikkanen et al., 2004; Dunker et al., 2013). Cyanobacteria were found to potentially have strong lethal or inhibiting effect on phytoplankton. Affected algae through esterase activity suppression, photosynthesis and reduction of metabolic activity (Florence and Deborah, 1986; Zhang et al., 2007; Dunker et al., 2013; B-Béres et al., 2015). Microcystins (MCs) are thought to be responsible for some of these toxic effects, however, recent studies have demonstrated that also other secondary metabolites, which are extracellularly released, had allelopathic effects on other aquatic organisms (Suikkanen et al., 2004; B-Béres et al., 2015; Cirés and Ballot, 2016; Harke et al., 2016).

M. aeruginosa, one of the most common and harmful bloom-forming cyanobacterial species (e.g., Fogg, 1969; Figueiredo et al., 2004), is also known for such allelopathic effects. For example, Bártová et al. (2011) reported that extracts and exudates of *M. aeruginosa* PCC7806 induced oxidative stress-related responses in a green algae. Allelopathic effects of *M. aeruginosa* (toxic strain

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http://dx.doi.org/10.1016/j.hal.2016.11.010
1568-9883/

FACHB-905) exudates were confirmed important for disrupting germination and seedling growth of submerged macrophytes (Zheng et al., 2013; Xu et al., 2016), potentially contributing to the disappearance of an endangered macrophyte species in Dianchi Lake (Xu et al., 2015). Xu et al. (2015, 2016) for the first time reported that these allelopathic effects of *M. aeruginosa* on submerged macrophytes were dependent on the growth phase. Effects on macrophyte seedlings were strongly negative during the exponential growth phase, but turned into a promotion during the decline phase. Studies on the influence of the growth phase of *M. aeruginosa* on its allelopathic effects on algae, however, are lacking. We hypothesized that algae show a similarly differential response to exudates of the different *M. aeruginosa* growth phases.

To test this hypothesis, we added different concentrations of *M. aeruginosa* exudates of the exponential, stationary and decline phase to cultures of two green algae and a diatom species. In addition, we tested the effect of *M. aeruginosa* on different algae in co-culture experiments allowing for a continuous impact of allelochemicals released by the cyanobacterium.

2. Material and methods

2.1. Phytoplankton cultivation

Cultures of *M. aeruginosa* (FACHB-905, toxic), *Scenedesmus quadricauda* (FACHB-1297), *Chlorella pyrenoidosa* (FACHB-1220) and *Cyclotella meneghiniana* (FACHB-1182) were obtained from the Freshwater Algae Culture Collection of the Institution of Hydrobiology (FACHB-Collection) at the Chinese Academy of Sciences. They were axenically kept in a modified MIII nutrient solution (Körner and Nicklisch, 2002), which was also used for all experiments. Major nutrients (400 μmol L^{-1} Si, 500 μmol L^{-1} N, 50 μmol L^{-1} P, 10 μmol L^{-1} Fe) were assumed to be not limiting at the biomass densities used in our experiments (maximum final phytoplankton concentration $<$300 μg chl *a* L^{-1}). The prepared MIII nutrient solution was filtered through a glass fibre filter (0.45 μm) and put into a sterilized 500 mL flask. Cultures were grown semi-continuously (daily additions of fresh MIII nutrient solution) in a climate-controlled room (Dongnan, GXZ-380B, Ningbo, China) at 25 ± 1 °C in a 12:12 h light–dark cycle with light of 80 μmol quanta m^{-2} s^{-1} measured as photosynthetic photon flux density (PPFD) by a Quantum Meter (Spectrum Technology, Inc., USA), manually shaken twice daily.

2.2. Experimental design

Two experiments were designed to test the allelopathic effects of *M. aeruginosa* on two green algae (*S. quadricauda, C. pyrenoidosa)* and the diatom *C. meneghiniana.* In Exp. 1, exudates of three different growth phases were added at different concentrations to the three algae cultures, while in Exp. 2, *M. aeruginosa* was co-cultured with each of the algae test species.

2.2.1. Experiment 1: Addition of M. aeruginosa exudates

In Exp. 1, exudates from the exponential (EP), stationary (SP) and decline phase (DP) of *M. aeruginosa* were added to cultures of *S. quadricauda, C. pyrenoidosa* and *C. meneghiniana.* Cells of *M. aeruginosa* were manually counted by hemocytometer with a light microscope (Olympus, Japan) every day to confirm their growth stage (EP, SP and DP). The initial density of the *M. aeruginosa* culture was $1.9*10^6$ cells L^{-1}. Exudates of the EP were harvested at a concentration of $5*10^7$ cells L^{-1}. This density is comparable to reported densities of cyanobacteria during blooming periods in Lake Dianchi ranging from $2*10^7$ cells L^{-1} to $4*10^8$ cells L^{-1} (Wan et al., 2008). Subsequently, these cultures were divided into two parts: one for obtaining the exudates of EP while the other remained without supplement to enter the stationary phase. Half of the SP cultures of *M. aeruginosa* were harvested for obtaining exudates of the SP (at a concentration of $3*10^8$ cells L^{-1}, about 30 days after inoculation), while the rest was kept till reaching the decline phase. Exudates of DP cultures were harvested two weeks after the start of the decline phase (more than 6 months after starting the cultivation) at a concentration of $2.3*10^9$ cells L^{-1}. To obtain cyanobacterial exudates, *M. aeruginosa* cultures from different growth stages were centrifuged with 6000 r min^{-1} for 10 min and subsequent filtration of the supernatant through a glass fibre filter (0.45 μm) under axenic conditions. *M. aeruginosa* exudates were then diluted into five concentrations (100%, 75%, 50%, 25%, and 0% (control)) using nutrient solution. Concentrations of nitrate and phosphate were adjusted to the same level as in the MIII nutrient solution. The pH value of all media was modified to 8.0 using NaOH and HCl. At the end of the experiments, nitrate and phosphate concentrations were measured and showed no significant differences between treatments and control.

In exp. 1, the biomass of the target algae was determined by measuring their optical density (OD) at 680 nm (green algae) and 440 nm (diatom) with a spectrophotometer (cary 60, Agilent, USA). In addition, chl *a* concentrations were determined by measurements of F_0 fluorescence with a Phyto-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany) after dark adaptation for 20 min, as F_0 is closely correlated to chl *a* concentrations (Schreiber and Bilger, 1993; Chang et al., 2012). The initial OD of both tested green algae and the diatom was 0.04 and 0.02, respectively, in all treatments and controls. Cells were grown in flasks containing 100 mL of nutrient solution (controls, 0%), or exudates (25%, 50%, 75% or 100%) and incubated under the same conditions as mentioned in 2.1. 10 mL of each algae culture were sampled each day (and replaced by an equal volume of M III nutrient solution) to measure biomass and determine growth rates. 10 mL of fresh-made nutrient solution without or with exudates for controls and treatments, respectively, was added into each flask every day. The test lasted for 3 days with 4 replicates for each treatment and each control. Inhibition or enhancement of algae biomass relative to the control (*I*) was calculated based on OD values and chl *a* as:

$$I\,(\%) = (1\text{-}M/N) \times 100 \quad (1)$$

with *N* representing the control data and *M* the exudate treatment data.

2.2.2. Experiment 2: Co-culture test

In exp. 2, *M. aeruginosa* was co-cultured with *S. quadricauda, C. pyrenoidosa* and *C. meneghiniana*, respectively, with an initial ratio of *M. aeruginosa* to other phytoplankton species of 4:0, 3:1, 1:1, 1:3 and 0:4 (based on chl *a* measurements, total chl *a* concentrations: 60 μg L^{-1}), and total volume is 100 mL for each sample. The mixed cultures were incubated in a climate controlled room under the same conditions as described in 2.1.

Chl *a* concentrations of each species were determined in subsamples after 24, 48 and 72 h by a Phyto-PAM fluorometer which uses four different excitation wavelengths and thus allows a distinction between diatoms, green algae and cyanobacteria in mixed cultures (Schreiber and Bilger, 1993). Chl *a* concentrations, measured at the beginning (C_0) and after t_n (n = 1, 2, 3 days), were used to calculate the growth rates for each species each day as:

$$\mu = (\ln C_n\text{-}\ln C_0)/(t_n\text{-}t_0) \quad (2)$$

2.3. Statistical analyses

Data were expressed as means?standard deviation. In exp. 1, the effect of the different concentrations of exudates and the growth