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Evaluating the effects of allelochemical ferulic acid on *Microcystis aeruginosa* by pulse-amplitude-modulated (PAM) fluorometry and flow cytometry

Rui Wang [a], Ming Hua [b], Yang Yu [c], Min Zhang [c], Qi-Ming Xian [b], Da-Qiang Yin [a, *]

[a] State Key Laboratory of Pollution Control and Resource Reuse, College of Environmental Science and Engineering, Tongji University, Shanghai 200092, PR China
[b] State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing 210093, PR China
[c] Nanjing Institute of Geography and Limnology, Chinese Academy of Science, 210093, Jiangsu, PR China

HIGHLIGHTS

- Ferulic acid exposure inhibited the growth of blue-green algae *Microcystis aeruginosa.*
- Allelopathic effects were evaluated by PAM and flow cytometry as diagnostic tools.
- Ferulic acid inhibited the photosynthetic activities of *M. aeruginosa.*
- Ferulic acid inhibited the membrane potential and esterase activity of *M. aeruginosa.*
- Membrane potential and esterase activity were identified as two sensitive variables.

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ABSTRACT

We investigated the effects of allelochemical ferulic acid (FA) on a series of physiological and biochemical processes of blue-green algae *Microcystis aeruginosa*, in order to find sensitive diagnostic variables for allelopathic effects. Algal cell density was significantly suppressed by FA (0.31–5.17 mM) only after 48 h exposure. Inhibitions of photosynthetic parameters (F_v/F_m and F_v'/F_m') occurred more rapidly than cell growth, and the stimulation of non-photochemical quenching was observed as a feed-back mechanisms induced by photosystem II blockage, determining by PAM fluorometry. Inhibitions on esterase activity, membrane potential and integrity, as well as disturbance on cell size, were all detected by flow cytometry with specific fluorescent markers, although exhibiting varied sensitivities. Membrane potential and esterase activity were identified as the most sensitive parameters (with relatively lower EC_{50} values), and responded more rapidly (significantly inhibited only after 8 h exposure) than photosynthetic parameters and cell growth, thus may be the primary responses of cyanobacteria to FA exposure. The use of PAM fluorometry and flow cytometry for rapid assessment of those sensitive variables may contribute to future mechanistic studies of allolepathic effects on phytoplankton.

1. Introduction

Numerous aquatic macrophyte species have been shown to suppress phytoplankton growth via releasing various allelopathically active compounds (Nakai et al., 1999; Gross, 2003). The allelopathic interference has been proposed as an important mechanism to stabilize the clear-water states in macrophytes-dominated shallow lakes (Hilt and Gross, 2008). Most studies on the effects of allelochemicals use plants extracts or purified plant compounds, however such procedures could not reflect the natural lake conditions and often exhibit synergistic effects of mixed allelochemicals (Nakai et al., 2000; Zhu et al., 2010). Field studies and co-culturing experiments are more realistic, but hard to differentiate the allelopathic inhibition versus other competitive interactions (i.e., shading, sediment resuspension, nutrients/light competition, Mulderij et al., 2007; Nemoto and Fukuhara, 2012).

* Corresponding author. College of Environmental Science and Engineering, Tongji University, 1239 Siping Road, Shanghai 200092, China.
E-mail address: yindq@tongji.edu.cn (D.-Q. Yin).

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Such confounding factors could be prevented by directly short-term exposure of phytoplankton to a single allelochemical, however, such study would require sensitive variables that could respond rapidly to allelochemicals.

Phenols and polyphenols are the most common class of allelochemicals released by macrophytes. For example, the extract of *Myriophyllum spicatum* (a common submerged macrophyte) significantly inhibited algal growth, containing 14 phenols and polyphenols, e.g., ferulic, caffeic, ellagic and gallic acids (Planas et al., 1981). It has been noticed that the sensitivities of allelopathic effects varied greatly with the phytoplankton groups, that cyanobacteria generally appeared to be more susceptible to allelochemicals than green algae (Hilt and Gross, 2008). Besides, varied allelochemicals exhibited distinct inhibition effects on phytoplankton, e.g., polyphenols generally induced stronger inhibition on cyanobacteria than those phenols with methoxy groups (summarized by Nakai et al., 2001). The detection of various observation variables would help the evaluation of such great variation. Allelochemicals can interfere with many processes of the target organisms (as postulated by Reigosa et al., 1999), among which two physiological processes have been widely identified as the most common modes of action on phytoplankton: (I) the inhibition of photosynthesis (especially photosystem II), likely via inhibiting the photosynthetic electron transport (Körner and Nicklisch, 2002; Zhu et al., 2010) and (II) inhibition of enzyme activities (e.g., alkaline phosphatase), since hydrolysable polyphenols are expected to pass the dialysis membranes and effectively complex with proteins (Gross et al., 1996). Studies on other physiological and/or biochemical responses which could respond more rapidly and sensitively are lacking.

Ferulic acid (4-Hydroxy-3-methoxycinnamic acid, FA) is widely recognized as an allelochemical in terrestrial ecosystem, which can be released from plant roots (e.g., vegetables, fruits) thus involve in the allelopathic interactions between plants and soil microbial communities (Zhou and Wu, 2012; Caspersen et al., 2000). In aquatic ecosystem, FA could be released by submerged and floating-leaved macrophytes (Pip, 1992), as well as algae (López et al., 2011). Besides, FA levels in polluted systems could also be elevated via anthropogenic discharges, since FA is widely used in medicines, cosmetics and health foods due to its antioxidant activities (Mancuso and Santangelo, 2014). Nevertheless, whether FA can cause an effective inhibition on phytoplankton and the possible allelopathic mechanism was seldom studied. In the present study, we tried to investigate the effects of ferulic acid on *Microcystis aeruginosa* (the most common bloom-forming cyanobacteria, known to be sensitive to polyphenolic allelochemicals, Hilt and Gross, 2008) after short-term exposure. Two diagnostic technologies for ecotoxicity testing were applied to evaluate multiple variables of the exposed algae. Flow cytometry, a rapid method for the quantitative measurement of the cellular algal characteristics by determining the fluorescence signals (derived via direct autofluorescence, or mediated after staining with specific fluorescence markers, Stauber et al., 2002), was used to assess the metabolic status of cells (i.e., esterase activity) and membrane characteristics (i.e., membrane integrity and membrane potential). Pulse amplitude modulated (PAM) fluorometry, a rapid and sensitive tool for assessing photosynthetic activity in microalgae (Juneau et al., 2001), was applied to evaluate the photosynthetic performance of *M. aeruginosa* after FA exposure.

The aims of the present study were (I) to investigate whether the effects of FA on *M. aeruginosa* were detectable after short term exposure using PAM fluorometry and flow cytometry, and (II) to identify the sensitive variables in *M. aeruginosa* response to short-term exposure of FA by comparing their modelled EC_{50} values. Our study results could provide insight into the interaction mechanism between allelochemical and phytoplankton.

2. Materials and methods

2.1. Algal culture and exposure

Cultures of the cyanobacteria *M. aeruginosa* (non-microcystin producing) used in this study were obtained from the Environment Biology Laboratory at Nanjing University, China. MA medium, reported by Ichimura (1979), was used for the algal pre-culture and experiments. The MA medium was prepared using analytical grade chemicals dissolved in Milli-Q water, and the composition (per liter) was as follows: $Ca(NO_3)\cdot 4H_2O$, 50 mg; KNO_3, 100 mg; $NaNO_3$, 50 mg; Na_2SO_4, 40 mg; $MgCl_2\cdot 6H_2O$, 50 mg; β-sodium glycerophosphate, 100 mg; P(IV) metals, 5 ml; H_3BO_3, 20 mg; Bicine, 500 mg (pH 8.6). Batch cultures of *M. aeruginosa* were incubated in the MA medium at a temperature of 25 ± 0.5 °C, under a well-controlled 12:12 light–dark cycle (with 1500 lux of illumination). The exponentially growing cells were used as inoculum for the start of experiments.

Ferulic acid (99.5%) used in this study was purchased from Sigma Ltd. Co., and stored at -4 °C in dark. To obtain the dose–response effects of FA on *M. aeruginosa*, the exposure medium were prepared by adding FA in 250 mL flasks containing 100 mL MA medium at a series of concentrations: 0.31, 0.47, 0.70, 1.04, 1.56, 2.32, 3.47, 5.17 mM. At the beginning of exposure, *M. aeruginosa* was immediately inoculated into the freshly prepared exposure medium, with an initial cell density of 5.0×10^5 cells/mL. Exposure without adding ferulic acid was set as control. All the cultures were shaken every day to avoid cell aggregation. Each treatment has five replicates. Specific volumes of algal suspensions were taken at time intervals (8, 24, 48 and 96 h) and filtrated prior to further measurements. The cell density was determined based on the linear relationship between cell counting (using a hemocytometer under microscope) and optical density (measured at 680 nm using a spectrophotometer). We observed that the detected FA concentrations in the medium slightly decreased with exposure time (less than 10%, in all treatments), and the variations between nominal concentration and detected concentration were less than 5% at the start of experiment. To make our results much easier to understand, nominal concentrations were used to indicate the exposure level.

2.2. Flow cytometric measurements

Flow cytometric measurements (FCM) were conducted with a FACS Vantage SE flow cytometer (Becton–Dickinson, USA) equipped with a dual-laser bench and optics (Coherent Innova 70-4 laser emitting at 488 nm). Fluorescence was collected at specific range of wavelengths by two-color photomultiplier tubes with fluorescence emission filters, including: SSC/FSC FL1 530/30 nm (AMG = 8), green fluorescence; FL2 580/42 nm, orange fluorescence. Dead cells and non-algal particles were excluded from the analysis by gating on SSC/FL3. Acquisition was made using the pulse height and log mode for all variables. The program Cell-Quest from Becton–Dickinson was used to collect and analyze these signals.

Fluorescein diacetate (FDA, Sigma F-7378) was used as the probe to assess esterase activity (Franklin et al., 2001), a sensitive endpoint in algal toxicity (Yu et al., 2007). The cell staining was performed by treating 1 mL algal suspension (5×10^5 cells/mL) with 25 μM FDA (working solution dissolved in acetone), at pH of 8.0, incubating for 8 min at room temperature. Acetone at the tested concentration has no toxicity on the cyanobacteria (Yu et al., 2007). Fluorescein was detected on the flow cytometer in the FL1 channel (green fluorescence).

A fluorescent lipophilic cationic dye 3,3′-