Chemosphere 186 (2017) 527–534

Contents lists available at ScienceDirect

Chemosphere

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

Effects of an allelochemical in *Phaeodactylum tricornutum* filtrate on *Heterosigma akashiwo*: Morphological, physiological and growth effects

Rui Wang [a, b], Qiaona Xue [a], Jiangtao Wang [a, *], Liju Tan [a], Qingchun Zhang [c], Yue Zhao [c], Donald M. Anderson [d]

[a] Key Laboratory of Marine Chemistry Theory and Technology, Ministry of Education, Ocean University of China, Qingdao 266100, China
[b] Shenzhen Key Laboratory for the Sustainable Use of Marine Biodiversity, Research Centre for the Oceans and Human Health, City University of Hong Kong Shenzhen Research Institute, Shenzhen 518057, China
[c] Key Laboratory of Marine Ecology and Environmental Sciences, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China
[d] Biology Department, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, United States

HIGHLIGHTS

- An allelochemical isolated from filtrate of *Phaeodactylum tricornutum* inhibited the growth of *Heterosigma akashiwo*.
- Allelopathic effects were evaluated using the SEM and FCM as convenient diagnostic approaches.
- The allelochemical damaged the membrane integrity and affected the esterase activity of *H. akashiwo*.
- Esterase activity was the most sensitive indicator of the impacts of the allelochemical.

ARTICLE INFO

Article history:
Received 24 April 2017
Received in revised form
16 July 2017
Accepted 7 August 2017
Available online 7 August 2017

Handling Editor: Frederic Leusch

Keywords:
Allelochemical
Phaeodactylum tricornutum
Heterosigma akashiwo
Flow cytometry
Physiological characters

ABSTRACT

The effects of an allelochemical extracted from the culture filtrate of diatom *Phaeodactylum tricornutum* on the raphidophyte *Heterosigma akashiwo* were investigated using a series of morphological, physiological and biochemical characters. Growth experiments showed that *H. akashiwo* was significantly inhibited immediately after exposure to the allelochemical, with many cells rapidly dying and lysing based on microscopic observation. The effects of the allelochemical on the surviving cells were explored using Scanning Electron Microscopy (SEM) and Flow cytometry (FCM), the latter by examination of a suite of physiological parameters (membrane integrity, esterase activity, chlorophyll-*a* content, membrane potential). The results demonstrate that the membrane of *H. akashiwo* was attacked by the allelochemical directly, causing cell membrane breakage and loss of integrity. Esterase activity was the most sensitive indicator of the impacts of the allelochemical. Membrane potential and chlorophyll-*a* content both showed significant decreases following exposure of the *Heterosigma* cells to high concentrations of the allelochemical for 5 and 6 days. Both were affected, but the membrane potential response was more gradual compared to other effects. The cell size of *H. akashiwo* did not change compared with the control group. The surviving cells were able to continue to grow and in a few days, re-establish a successful culture, even in the presence of residual allelochemical, suggesting either development of cellular resistance, or the degradation of the chemical.

1. Introduction

Harmful algal blooms (HABs) occur frequently in marine coastal areas and freshwater ecosystems worldwide, causing serious consequences on the environment, aquaculture industries and human health (Anderson, 1997; Horner et al., 1997; Anderson et al., 2012; Dorantes-Aranda et al., 2015). One of the factors thought to be important in phytoplankton competition for resources and community dynamics is allelopathy - the release of secondary metabolites into an organism's surroundings, thereby affecting the

* Corresponding author.
E-mail address: jtwang@ouc.edu.cn (J. Wang).

http://dx.doi.org/10.1016/j.chemosphere.2017.08.024
0045-6535/

growth or viability of co-occurring organisms (Rice, 1984; Legrand et al., 2003; Roy et al., 2006; Yang et al., 2014).

The production of allelochemicals among dinoflagellates, diatoms, chrysophytes and cyanobacteria has been reported in many marine systems (e.g., Sukenik et al., 2002; Gross, 2003; Legrand et al., 2003; Irfanullah and Moss, 2005; Granéli et al., 2012), and allelopathic interference has been proposed as an important mechanism to stabilize the clear-water states for macrophytes dominating in shallow lakes (Hilt and Gross, 2008; Wang et al., 2016a). The mechanisms through which allelochemicals are released by phytoplankton and impact on other phytoplankton remain unclear in many marine ecosystems. Allelochemicals influence multiple cell functions including cell division, metabolism, photosynthesis, respiration, and enzyme activity (Duke, 2003; Singh and Thapar, 2003; Belz and Hurle, 2004). For example, *Chlorella vulgaris* cell membrane was detached from the cell wall after exposure to *N*-phenyl-2-naphthylamine (Qian et al., 2009), an allelochemical isolated from root exudates of water hyacinth (*Eichhornis crassipes*) (Sun et al., 1993). The inhibition of photosynthesis (especially photosystem II) and the inhibition of enzyme activities (e.g., alkaline phosphatase) were also identified as common modes of actions for allelochemicals (Gross et al., 1996; Körner and Nicklisch, 2002; Zhu et al., 2010; Wang et al., 2016a). Enzyme activity is increasingly measured in microalgae (e.g., peroxidases, b-galactosidases, esterases) with a rapid and sensitive endpoint (Peterson and Stauber, 1996; Blaise and Ménard, 1998; Franklin et al., 2001; Eigemann et al., 2013). Ethyl 2-methyl acetoacetate (EMA) isolated from *Phragmites communis* had impacts on respiration and photosynthesis of *Microcystis aeruginosa* (Li et al., 2007).

One of the challenges in this field of investigation is in characterizing allelochemical effects beyond simple growth rate reductions. In recent years, flow cytometry (FCM) has emerged as a rapid and highly efficient analytical method to measure these types of changes in microalgae (e.g., Xiao et al., 2010, 2011; 2014). The application of FCM provides a convenient diagnostic approach for understanding and quantifying allelopathic interactions (Rioboo et al., 2009). Here we use FCM method, together with scanning electron microscopy (SEM) to characterize the morphological, physiological and growth effects of an allelochemical produced by the diatom *Phaeodactylum tricornutum* on the raphidophyte *Heterosigma akashiwo*.

Heterosigma akashiwo is a common, highly successful bloom - forming species responsible for many fish-killing blooms throughout the world, typically in nearly mono-specific blooms at cell densities that lead to the appearance of discolored water (e.g., red tides) (Smayda et al., 1998). The production of chemical compounds (allelochemicals) by *H. akashiwo* is well established as a strategy to inhibit the growth of other species of co-occurring microalgae (Yamasaki et al., 2007; Qiu et al., 2012). In our study, *Phaeodactylum tricornutum* has hard siliceous walls with a strong resistance to certain types of allelochemicals, particularly to those that act at the cell surface. We successfully demonstrate that *Phaeodactylum* has evolved an ability to release allelochemicals that can dramatically affect organisms like *Heterosigma* (Wang et al., 2016b), whereas the compounds released by *Heterosigma* do not appreciably affect *Phaeodactylum*, even though they have been shown to inhibit other diatoms. A putative allelochemical (a type of glycinamide compound) was isolated from the filtrate of *P. tricornutum* (Wang et al., 2016b), however, details of the growth inhibition and mechanism of action are lacking. In the present study, we document responses of *H. akashiwo* cells to the allelochemical present in *P. tricornutum* filtrate. These results provide new insight into the mechanism of allelochemicals in marine ecosystems.

2. Materials and methods

2.1. Algal culture and isolation of allelochemical from P. tricornutum filtrate

Phaeodactylum tricornutum and *Heterosigma akashiwo* were obtained from the Algal Center of Key Laboratory of Marine Chemistry Theory and Technology, Ocean University of China. The microalgae were cultivated in f/2 medium (Guillard, 1975) prepared using autoclaved seawater (filtered through 0.45 μm Millipore membranes) from Jiaozhou Bay of China. Cultures were grown at (20 ± 1) °C with a 12/12-h light/dark cycle. Illumination was provided by cool white filament lamps at 70 μmol $m^{-2}s^{-1}$. All glassware was acid-soaked, cleaned with milli-Q water, and autoclaved. Cultures were gently shaken twice manually every day to avoid wall growth and prevent the sedimentation of algae.

A 27-L culture was maintained in a transparent polyethylene container until late exponential phase. The filtrate was obtained by centrifugation (1814.4×*g*, 15 min). A small number of cells from the pellet were observed under the microscope (Leica DM4000B, Germany) after centrifugation to demonstrate that the cells remained intact. The supernatant was then filtered through a 0.22 μm membrane and the filtrate extracted with ethyl acetate three times in succession. The extracts were pooled and evaporated to dryness using a rotary vacuum evaporator (Beijing Bo Kang Laboratory Instruments Medical Co., Ltd.) under reduced pressure at 40 °C. The extract was diluted to 10 mL with DMSO and stored at 4 °C.

The ethyl acetate extract from the filtrate of *Phaeodactylum tricornutum* was purified using HPLC with 99 μL injection volume (repeated 10 times) according to the elution times of chromatographic peaks. Nine isolated fractions were dried under N_2 and the residues diluted with 1.0 mL DMSO respectively. The putative allelochemical was obtained from fraction VI (Wang et al., 2016b). The isolated allelochemical was dissolved in 1 mL DMSO to the same concentration of crude ethyl acetate extract before the HPLC separation, and this was then used for all bioassays in the present study.

2.2. Sample preparation for SEM

Heterosigma akashiwo was cultured for 4 d with an initial cell density of 1.0×10^4 cells mL^{-1}. 37 μL DMSO solution was then added into the culture medium of *H. akashiwo*. After 4 d of exposure to the DMSO solution, algal cells were collected by centrifugation (1814.4×*g*, 10 min) and fixed overnight with 2.5% glutaraldehyde at 4 °C. Samples were washed with 0.1 mol L^{-1} phosphate buffer solution (PBS, pH = 7.4) and centrifuged (1814.4×*g*, 10 min) three times, then the supernatant was discarded. Algal cells were fixed with 1% osmium tetroxide at 4 °C for 1 h, washed by 0.1 mol L^{-1} PBS (pH = 7.4), then centrifuged three times, discarding the supernatant. Algal samples were dehydrated with 30%, 50%, 70%, 80%, 90%, 95% and 100% alcohol solutions for 20 min. Samples were then fixed in tert butyl alcohol and freeze-dried for final SEM (Hitachi, Japan) observation after dehydration.

2.3. Flow cytometric measurements

Flow cytometry was conducted with a BD Accuri C6 flow cytometer (Becton Dickinson, USA) equipped with a blue and red laser (488 nm emission), two light scatter detectors, and four fluorescence detectors with optical filters, including FL1 530/15 nm; FL2 585/20 nm; FL3>670 nm and FL4 675/12.5 nm. The program C Flow Plus from Becton Dickinson was used to collect and analyze signals.

All added concentrations of allelochemical were divided into