



The green macroalga, *Ulva lactuca*, inhibits the growth of seven common harmful algal bloom species via allelopathy

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ABSTRACT

Harmful algal blooms (HABs) are a significant threat to fisheries, public health, and economies around the world, and both HABs and macroalgae are often promoted by nutrient loading. We report on experiments examining the effects of the macroalga, *Ulva lactuca*, collected from estuaries of NY, USA, on the growth of seven common HAB species: *Aureococcus anophagefferens*, *Chattonella marina*, *Cochlodinium polykrikoides*, *Karlodinium veneticum*, *Karenia brevis*, *Prorocentrum minimum* and *Pseudo-Nitzschia multiseries*. Fresh thalli of *U. lactuca* added at environmentally realistic levels (mg L^{-1}) were capable of lysing or strongly inhibiting the growth of all seven HAB species in a dose-dependent manner within controlled laboratory experiments during which high nutrient levels, low bacterial levels, and common pH levels among treatments and controls were maintained. The dramatic allelopathic effects of extracts of dried and powdered *U. lactuca* with and without post-extraction heat treatment on the HAB species demonstrated that *U. lactuca* contains heat-stable allelochemicals that play a major role in the observed allelopathic effects. The addition of live *U. lactuca* thalli in bottle and mesocosm experiments conducted in the field during blooms of *A. anophagefferens* ('brown tide'; $>10^5$ cells mL^{-1}) consistently yielded a significant ($p < 0.05$) and often large ($>50\%$) reduction in cell densities in ~ 48 h. Our findings combined with the well-known nutrient removal capacity of seaweeds collectively suggest that the use of macroalgae may be a promising mitigation strategy for HABs in coastal ecosystems.

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1. Introduction

Harmful algal blooms (HABs) are a significant threat to fisheries, public health, and economies around the world. The frequency and impacts of HABs have intensified in recent decades and anthropogenic nutrient loading has been implicated in this expansion (Anderson et al., 2008; Heisler et al., 2008). Another group of autotrophs promoted by eutrophication in coastal ecosystems is macroalgae (Valiela et al., 1997; Valiela and Cole, 2002; Valiela, 2006). Many species of macroalgae are capable of rapid growth in the presence of high nutrient concentration and have a high assimilative capacity for nutrients thus act as nutrient sinks (Valiela et al., 1997; Neori et al., 2004; Zertuche-González et al., 2009). Like HABs, the overgrowth of macroalgae can be also considered harmful, as they can outcompete and replace seagrass beds, cover other critical benthic habitats, and promote diel hypoxia in estuaries (Valiela et al., 1997; Valiela and Cole, 2002; Valiela, 2006; Liu et al., 2009).

Recent studies, largely in Asia, have demonstrated that macroalgae can have strong growth-inhibiting effects on phytoplankton in general, and multiple HAB species, in particular. Multiple species of the green macroalgal genus, *Ulva*, including *Ulva fasciata*, *Ulva pertusa*, and *Ulva linza* have displayed the ability to cause rapid lysis of *Prorocentrum micans*, *Prorocentrum donghaiense*, *Heterosigma akashiwo* (Jin and Dong, 2003; Jin et al., 2005; Nan et al., 2004, 2008; R.J. Wang et al., 2007; Y. Wang et al., 2007) and to reduce the growth of *Alexandrium tamarense* and *Chaetoceros gracile* (Nan et al., 2004). The red coralline macroalga, *Corallina pilulifera*, has displayed algicidal activity against several harmful algae including *Cochlodinium polykrikoides*, *Karenia mikimotoi*, *Akashiwo sanguinea*, *A. tamarense*, and *H. akashiwo* (Jeong et al., 2000; R.J. Wang et al., 2007). Other macroalgae that have been shown to inhibit the growth of HAB species include *Ecklonia kurome*, *Gracilaria lemaneiformis*, and *Sargassum thunbergii* (Koki et al., 2003; Liu et al., 2006; Lu et al., 2008; R.J. Wang et al., 2007; Y. Wang et al., 2007). The impacts of macroalgae on HABs have been observed from both co-culturing and, in limited cases, from administering macroalgae extracts (Jeong et al., 2000; Jin et al., 2005; R.J. Wang et al., 2007). Two studies have demonstrated that polyunsaturated fatty acids isolated from *U. fasciata* and *U. pertusa* have effects on HABs similar to those observed for extracts and whole macroalgae,

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suggesting these compounds could be active agents against the HAB species (Alamsjah et al., 2005, 2008). Studies unambiguously demonstrating the allelopathic effects of macroalgae on HABs, however, have been rare (Körner and Nicklisch, 2002; Gross et al., 2003, 2007). In addition, all of the research to date examining the effects of macroalgae on HABs has been conducted in Asia, although many macroalgae including species of *Ulva* are globally distributed along coastlines.

This study reports on the effects of live thalli and extracts of the macroalga, *Ulva lactuca*, isolated from NY, USA, on the growth of seven HAB species (*Aureococcus anophagefferens*, *Chattonella marina*, *Pseudo-Nitzschia multiseries*, *Cochlodinium polykrikoides*, *Karenia brevis*, *Karlodinium veneticum*, and *Prorocentrum minimum*) within controlled laboratory, field, and mesocosms experiments. These seven species commonly form HABs in coastal waters in the US and around the world. Results demonstrate that allelochemicals produced by *U. lactuca* are capable of lysing or strongly inhibiting these HABs and thus that this macroalga has the potential to impact the occurrence of HABs.

2. Materials and methods

2.1. Microalgal cultures (target HAB species)

The targeted HAB species included in the study were 4 species of dinoflagellates (*P. minimum*, *C. polykrikoides*, *K. veneticum*, and *K. brevis*), one species of the 'brown tide' pelagophyte (*Anophagefferens anophagefferens*), a species of the toxigenic diatom *P. multiseries*, and one species of raphidophyte, *C. marina*. The cultures of *P. minimum* (strain CCMP696), *K. brevis* CCMP2228, and *A. anophagefferens* (strains CCMP1984 and CCMP1707) were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Maine, USA). The culture of *C. marina* (strain Chatt1) from Singapore coastal water was kindly provided by M.J. Holmes (National University of Singapore), the diatom *P. multiseries* CLNN21 was isolated from the Bay of Fundy, Canada and kindly provided by Steve Bates (Fisheries and Oceans Canada), and the cultures of *C. polykrikoides* (strain CP1) and *K. veneticum* (strain FR-6) were isolated by YZ Tang from Flanders Bay and the Forge River, respectively, two estuaries on Long Island, NY, USA; their identity has been confirmed via genetic sequencing (Tang et al., 2010). The three cultures of *P. minimum* CCMP696 and *A. anophagefferens* strains CCMP1984 and CCMP1707 were also initially isolated from estuarine waters of Long Island, NY, USA.

All the microalgal cultures except *P. multiseries* were maintained in GSe medium (G medium supplemented with 1×10^{-8} M selenium) (Doblin et al., 1999), made with autoclaved and sterile filtered (0.22 μm) seawater with a salinity of ~ 30 PSU. The diatom *P. multiseries* was maintained in GSe medium supplemented with Si at the same concentration in f/2 medium. Cultures were maintained at 21 °C in an incubator with a 12:12 h light:dark cycle, illuminated by a bank of fluorescent lights that provided a light intensity of $\sim 100 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ to cultures. An antibiotic solution (a mixture of 10,000 I.U. penicillin and 10,000 $\mu\text{g mL}^{-1}$ streptomycin; Mediatech, Inc., Hemdon, VA) was added into the medium of all cultures except for *K. veneticum* FR-6 immediately before inoculation, with a final concentration of 1% to minimize contamination by bacteria. This antibiotic mixture had no negative effects on the growth and survivorship of these microalgae although *K. veneticum* cannot survive this antibiotic solution (Tang et al., 2010). Periodic DAPI-staining of cultures was performed and confirmed an absence of bacteria in cultures. An experiment using *A. anophagefferens* CCMP1984 as target species was performed with and without addition of antibiotics solution (final 1%) into culture medium to assess the effect of the macroalga on HAB

species in the presence of bacteria. All culture transfers were conducted within a sterile, class-100 laminar flow hood.

2.2. Collection, treatment, and maintenance of macroalgae

Fresh thalli of the macroalga *U. lactuca* were collected from the Old Fort Pond, a shallow estuary on eastern Long Island, NY. Thalli were liberally washed with 0.2 μm filtered seawater and then placed in GSe medium containing 4% antibiotics mixture overnight in an incubator. The 4% antibiotics-containing GSe medium was then changed several times over several days. Vegetative culture of macroalga was maintained in vessels containing GSe medium and 1% antibiotics under the conditions described above for cultures of HAB species. Periodic DAPI-staining of cultures was performed and confirmed an absence of bacteria in cultures.

2.3. Laboratory experiments

2.3.1. Experiments using fresh thalli of *U. lactuca*

The thalli of *U. lactuca* were cut into measured, rectangular pieces of 1 cm $\times$ 1 cm, 2 cm $\times$ 2 cm, 3 cm $\times$ 3 cm, and 4 cm $\times$ 4 cm and administered into 250-mL flasks containing 50 mL cultures of the targeted species, *A. anophagefferens*, *C. marina*, *P. multiseries*, *P. minimum*, *C. polykrikoides*, *K. veneticum*, and *K. brevis*. Care was taken to excise the middle sections of *U. lactuca*, ensuring the vegetative portion of thalli were used in experiments. Consequently, thalli never formed or released gametes during any experiment reported here. The placement of 1 cm $\times$ 1 cm *U. lactuca* in 50 mL culture is equivalent to single layer of macroalgae covering the bottom of a 0.5 m deep water, a density that is often exceeded in many shallow estuaries and tributaries across the northeast US coast (Valiela et al., 1997; Valiela and Cole, 2002; Valiela, 2006; authors pers. obs.). Triplicate thalli not used in experiments were immediately scanned using SigmaScan[®] software to confirm macroalgal area, and then dried in 65 °C for 24 h and weighed for dry weight. One cm² of wet *U. lactuca* was, on average, 2.27 ± 0.3 mg dry weight.

Densities of the HAB species used in experiments generally represented bloom and sometimes pre-bloom cell abundances, that were species specific (Gobler et al., 2005, 2007, 2008; Anderson et al., 2008). Control cultures without macroalgae were established at each phytoplankton cell density and all treatments were administered in triplicates. All flasks were incubated as described for culture maintenance. Growth of phytoplankton within flasks was monitored daily over 5 days via cell counts and chlorophyll fluorescence measurements using a TD-700 fluorometer (Turner Design). Flasks were also monitored for pH levels using high resolution pH paper (± 0.1 units) during experiments to assure cultures were not cross contaminated via a pH probe and with an electrode (Thermo Scientific Orion Star Series[™] Benchtop pH meter; ± 0.001 unit; calibrated prior each use with NIST traceable standards) at the end of experiments. The two methods were in good agreement ($\pm 5\%$).

Upon termination of the experiments, culture aliquots (5 mL) were removed, filtered through a combusted (450 °C for two h) glass fiber filter, and analyzed for nitrate using standard methods (Parsons et al., 1984); Measurements of light in larger experimental flasks with and without *U. lactuca* thalli at the experimental levels used scaled to larger volumes measured with a LiCor[®] PAR sensor, indicated that light intensity did not vary significantly among treatments at the levels administered as, in a manner consistent with an ecosystem setting, thalli were on the bottom of the flasks, while the light source was from above flasks.

All cultures used for experiments were in early or mid-exponential phase growth with high levels of ambient nutrients still present. Additions of GSe medium were made to all

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