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Effects of harmful algal blooms on competitors: Allelopathic mechanisms of the red tide dinoflagellate *Karenia brevis*

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Abstract

Because competitive interactions may have led to adaptations enabling bloom-forming phytoplankton to dominate pelagic communities, we explored the allelopathic effects of one red tide dinoflagellate, *Karenia brevis*, on competing phytoplankton species. Exposure to waterborne compounds from natural *K. brevis* blooms resulted in growth inhibition or death for four of five co-occurring species tested, whereas compounds exuded by *K. brevis* cultures suppressed three of these same competitors (the diatoms *Asterionellopsis glacialis* and *Skeletonema costatum* and the dinoflagellate *Prorocentrum minimum*) plus one additional species (the dinoflagellate *Akashiwo* cf. *sanguinea*) that was unaffected by bloom exudates. *K. brevis* exudates lowered photosynthetic efficiency and damaged cell membranes of competing phytoplankton, but had no effect on competitor esterase activity, nor did they limit competitor access to iron. Overall, during blooms, *K. brevis* exudes potent allelopathic compounds, competitors vary in their susceptibility to *K. brevis* allelopathy, and *K. brevis* may achieve nearly monospecific blooms by lowering the photosynthetic efficiency of competitor species and increasing competitor membrane permeability, eventually resulting in competitor growth suppression or death.

Because the composition of phytoplankton communities is determined by a wide variety of abiotic and biotic factors, the plankton environment has been used as a model system to understand species interactions and diversity through the lens of disturbance (Hutchinson 1961), predator–prey interactions (Leibold 1989), and resource competition (Tilman 1982). In addition to competing for limiting resources, phytoplankton may exclude each other more directly. The inhibition of competitors by the release of compounds, a process known as allelopathy, may be important in planktonic systems (reviewed in Legrand et al. 2003). Allelopathy has been hypothesized to play a role in species succession (Keating 1977), the formation of harmful algal blooms (Smayda 1997), and the establishment of invasive species (Figueredo et al. 2007).

Despite its likely importance, our understanding of allelopathy is still in the early stages. Allelopathy is difficult to conclusively demonstrate in the field, and responsible compounds have rarely been identified. Co-culturing experiments and observations of phytoplankton dynamics in the field have supported the possibility of allelopathy (e.g., Schmidt and Hansen 2001; Vardi et al. 2002), but have not definitively separated its effect from exploitative competition. Although lab experiments using high nutrient concentrations have helped to shed light on the process of allelopathy, their value has often been undermined by the use of whole-cell extracts rather than exudates (Freeburg et al. 1979), which places phytoplankton in contact with a suite of compounds not usually waterborne. We need to simultaneously better understand how allelopathy happens, and what its consequences are in the field (e.g., Fistarol et al. 2003).

Although allelopathic compounds remain mostly unidentified, mechanisms for allelopathy have been proposed in some cases. Possible modes of action include oxidative damage, loss of competitor motility, inhibition of photosynthesis, inhibition of enzymes, and membrane damage (reviewed in Legrand et al. 2003). For example, Vardi et al. (2002) found that the presence of the cyanobacterium *Microcystis* sp. caused a buildup of apoptosis-inducing reactive oxygen species in the competing dinoflagellate *Peridinium gatunense*. Further, compounds produced by two dinoflagellate species have been reported to cause loss of motility in competitor cells, although there are likely multiple mechanisms. Cell contact with dinoflagellate *Heterocapsa* sp. was required for a loss of competitor motility (Uchida et al. 1995), whereas cell-free filtrates of the toxic dinoflagellate *Alexandrium* spp. caused loss of motility in the heterotrophic dinoflagellate *Oxyrrhis marina* (Tillmann and John 2002).

Several studies have indicated that photosystem II (PSII) may be a target for allelopathy. Unknown compounds produced by the cyanobacterium *Trichormus doliolum* inhibited PSII in other cyanobacteria (Von Elert and Juttner 1997). However, a decrease in photosynthetic efficiency may be a symptom of allelopathy even if PSII is not the target. For example, Sukenik et al. (2002) found that compounds produced by the cyanobacterium *Micro-*

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cystis sp. inhibited carbonic anhydrase activity of the dinoflagellate *P. gatunense*, leading to CO_2 limitation and inhibition of photosynthesis. A decrease in PSII efficiency has also been reported for a variety of general cell stresses, including nutrient stress (Parkhill et al. 2001), temperature stress (Lesser and Gorbunov 2001), and metal toxicity (Miller-Morey and Van Dolah 2004).

In addition to carbonic anhydrase inhibition observed by Sukenik et al. (2002), other studies have reported enzyme inhibition as a mechanism for allelopathy. When tested as a pure compound, okadaic acid, produced by the dinoflagellate *Prorocentrum lima*, was shown to inhibit the growth of three microalgal species (Windust et al. 1996), perhaps because okadaic acid is a potent phosphatase inhibitior (Bialojan and Takai 1988). However, compounds other than okadaic acid appeared to be responsible for allelopathic effects of *P. lima* filtrates (Sugg and Van Dolah 1999). Microcystins, produced by the cyanobacterium *Microcystis aeruginosa*, were also shown to inhibit phosphatases (Dawson 1998), but no studies have demonstrated that microcystins are allelopathic at ecologically relevant concentrations (Babica et al. 2006). As with a decrease in photosynthetic efficiency, phytoplankton enzyme activity may be a general indicator of cell stress. For example, esterase activity in several species of marine and freshwater algae can be either enhanced or suppressed by copper toxicity (Franklin et al. 2001).

Damage to cell membranes is another proposed mechanism for allelopathy (Legrand et al. 2003). Microalgal compounds have been shown to damage red blood cell membranes, which suggests that competing phytoplankton could be similarly affected (Igarashi et al. 1998), and fatty acids potentially produced by microalgae have been shown to increase permeability of the plasma membranes of chlorophytes and cyanobacteria (Wu et al. 2006). The freshwater dinoflagellate *Peridinium aciculiferum* may cause membrane damage in competitor cells; Rengefors and Legrand (2001) observed that filtrates of *P. aciculiferum* caused blisters on the surface of *Rhodomonas lacustris* and eventually cell death. However, more research is needed to definitively establish that compounds exuded by phytoplankton at realistic waterborne concentrations cause membrane damage in competing phytoplankton.

Blooms of *Karenia brevis*, a red tide dinoflagellate occurring in the Gulf of Mexico and occasionally along the southeastern coast of the United States (Tester et al. 1991), cause ecosystem-wide effects, mostly due to the production of neurotoxic compounds (brevetoxins) that kill fish and accumulate in shellfish (Landsberg 2002). Like many dinoflagellates, *K. brevis* has a low nutrient affinity and growth rate compared with most diatoms and other phytoplankton taxa (Smayda 1997; Steidinger et al. 1998). This suggests that *K. brevis* does not achieve densities of millions of cells per liter (Steidinger and Haddad 1981), altering the phytoplankton community (West et al. 1996), by exploitation competition. One possible explanation for its occasional dominance is that *K. brevis* uses allelopathy to outcompete other phytoplankton species, as previously demonstrated using cultured strains of *K. brevis* (Kubanek et al. 2005).

Herein, we tested whether natural *K. brevis* blooms are allelopathic, and we investigated allelopathic mechanisms of action using both field samples and cultures of *K. brevis*. To better understand the mode of action of *K. brevis* allelopathy, we tested whether compounds exuded by *K. brevis* inhibit the photosynthetic efficiency or esterase activity of competitor species, damage competitor cell membranes, or limit competing species' access to iron.

Materials and methods

Collection of field samples—Three field samples of *K. brevis* (referred to as bloom samples) were collected approximately 10 m apart during a red tide at Long Boat Key Beach, near the city of Bradenton on the west coast of Florida, in September 2006. One natural seawater sample containing no *K. brevis* (referred to as a nonbloom sample) was collected from the nearby beach at Green Key, near New Port Richey, on the same day. *K. brevis* and other phytoplankton species were identified from samples stained with Lugol solution, counted by light microcopy using an Olympus IX-50 inverted microscope with a Palmer-Maloney settling chamber, and found to be present at $(3.2 \pm 1.8) \times 10^2$ cells mL^{-1} in the bloom samples, considered to be a medium-density bloom according to criteria set by the Florida Fish and Wildlife Research Institute (FWRI; http://research.myfwc.com/). Because the bloom began several weeks before samples were taken and persisted several weeks after collection, we designated the bloom to have been in maintenance stage (described in Steidinger et al. 1998). Although in our hands *K. brevis* could not be conclusively distinguished from morphologically similar congeners such as *Karenia mikimotoi*, reports by FWRI stated that *K. brevis* constituted the majority of *Karenia* spp. present during this bloom. Each bloom and nonbloom sample was maintained at approximately 25°C for 36 h with a 12-h light : dark (LD) cycle, then extracted using the method described below.

Phytoplankton culturing—Experiments were performed using five species of phytoplankton whose growth was previously shown to be suppressed by *K. brevis* live cells or filtrates (Kubanek et al. 2005). The following nonaxenic clones were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP): the dinoflagellates *Akashiwo* cf. *sanguinea* (CCMP 1740) and *Prorocentrum minimum* (CCMP 695), and the diatoms *Amphora* sp. (CCMP 129), *Asterionellopsis glacialis* (CCMP 137), and *Skeletonema costatum* (CCMP 775). All species are known to co-occur with *K. brevis* in the Gulf of Mexico, were isolated from the Gulf of Mexico or the Caribbean, and to tolerate similar light, nutrient, and temperature conditions. Cultures were maintained at 22°C with a 12-h LD cycle in a Percival incubator with Philips F32T8/TL741 Universal/Hi-Vision fluorescent bulbs mounted vertically, producing irradiance of 100–145 μmol m^{-2} s^{-1} throughout the incubator (measurement using Biospherical Instruments light meter model QSL2100), in F/2 + silicate media (Sigma-Aldrich) made with filtered Maine seawater (salinity 36). Cultures of *K. brevis* (CCMP

2228) used to generate extracellular extracts were grown in 2.5-liter Fernbach flasks in L1 + silicate media (CCMP) made with filtered Maine seawater (36). Growth curves were generated by visual cell count data using an Olympus IX-50 inverted microscope with a Palmer-Maloney settling chamber on culture samples preserved with Lugol solution.

Generation of extracellular extracts—Exuded organic compounds were extracted from each field sample and *K. brevis* culture by adding a mixture of three adsorbent resins (Diaion HP-20 [Supelco], Amberlite XAD-7-HP [Acros Organics], Amberlite XAD-16 [Supelco]) optimized for extraction of lipophilic compounds from aqueous media (Prince et al. 2006). Preliminary experiments in our lab confirmed that organic compounds with a range of polarity were extracted by this method, without lysing live *K. brevis* cells. We determined that a small proportion of inorganic nutrients contained in L1 media (6% of nitrate and 8% of phosphate) was also extracted from media using this method, but this did not cause an increase in the growth of any of the phytoplankton species relative to a solvent control (data not shown; $p = 0.31$–1.00 for all species). The ability to largely separate inorganic nutrients from organic exudates reduces the problem of nutrient artifacts in experiments, and is an advantage over traditional methods of generating cell-free filtrates. The resin was left in live field samples and cultures for 12–15 h, then recovered by slow filtration through 100-μm Nitex mesh and rinsed with deionized water. Lipophilic compounds were eluted from the resin with methanol followed by acetone, and solvents removed by rotary evaporation. Extracts of L1 + silicate media were generated in the same way, except without the presence of live cells. Brevetoxin B (PbTx-2) was quantified from *K. brevis* extracts by liquid chromatography–mass spectrometry as described in Kubanek et al. (2007). No other brevetoxins were present at concentrations above the detection limit.

Experiments—Experiment 1 tested whether compounds exuded by a bloom of *K. brevis* inhibit the growth of competing phytoplankton. We combined the three field sample extracts and determined that the combined extract contained 2.7 ng mL^{-1} PbTx-2 (i.e., from [3.2 $\pm$ 1.8] $\times$ 10^2 *K. brevis* cells mL^{-1}; representing an average of 8.5 pg of PbTx-2 exuded by each live *K. brevis* cell). We used a 48-h assay to test the effect of this combined *K. brevis* bloom extract on the growth of four competing phytoplankton species: *Amphora* sp., *A. glacialis, P. minimum*, and *S. costatum*, and a 96-h assay to test its effect on the slower-growing dinoflagellate *Akashiwo* cf. *sanguinea.* For all species except *A.* cf. *sanguinea*, a 200-μL inoculum of the competitor species and 2.8 mL of L1 + silicate media were added to each of 14 small culture tubes. Tubes with *A.* cf. *sanguinea* contained a 1.0-mL inoculum and 2.0 mL of L1 + silicate media. All tubes were placed haphazardly in an incubator at 22°C until competitor species reached exponential growth stage (approximately 3–5 d; cell densities of competitors in exponential growth ranged from 3.3 $\times$ 10^3 to 1.9 $\times$ 10^5 cells mL^{-1}). At that time, tubes were paired on the basis of similar in vivo fluorescence measurements and assigned arbitrarily to be either treatments or controls. To approximate natural concentrations of bloom and nonbloom waterborne compounds, we added an amount of extract equivalent to that generated from 3.0 mL of *K. brevis* bloom or nonbloom sample (dissolved in 3.3 μL of dimethyl sulfoxide [DMSO]; $n = 7$ replicate culture tubes for bloom treatment and nonbloom control). Using a Turner Designs TD-700 fluorometer calibrated with chlorophyll *a* (for all species used in these experiments, relationships between visual cell counts and fluorescence were linear; $r^2 = 0.75$–0.99), in vivo fluorescence (used as a proxy for relative cell concentrations) was assessed immediately before addition of extracts. Pairs were placed randomly in the incubator at 22°C after extract addition, and 48 or 96 h later the percentage change in in vivo fluorescence was determined by Eq. 1: $\frac{\text{final fluorescence} - \text{initial fluorescence}}{\text{initial fluorescence}} \times 100\%$. To normalize the percentage change in in vivo fluorescence by species we also calculated the percentage reduction in growth of treatments relative to controls using Eq. 2: $\frac{\Delta\text{control} - \Delta\text{treatment}}{\Delta\text{control}} \times 100\%$, where Δcontrol and Δtreatment are the percentage changes in in vivo fluorescence for the control and treatment, respectively, over the course of the experiment.

Because laboratory cultures provide a renewable source of allelopathic material to compare with bloom samples, we tested the effects of 16 independently generated extracts of *K. brevis* cultures in exponential growth stage on the growth of one susceptible competitor, *Asterionellopsis glacialis*, using the 48-h assay described above (with DMSO as control). The five *K. brevis* extracts that resulted in the strongest allelopathic effects against *A. glacialis* were pooled, and the effects of this pooled extract were tested on the growth of *Amphora* sp., *Akashiwo* cf. *sanguinea, Asterionellopsis glacialis, P. minimum*, and *S. costatum* (competitor cell density at onset of experiment ranged from 3.6 $\times$ 10^3 to 3.7 $\times$ 10^5 cells mL^{-1}), using 5 to 10 replicates with the assay method described above. The combined extract came from cultures with an average concentration of 3.02 $\times$ 10^4 *K. brevis* cells mL^{-1}, and contained 55 ng mL^{-1} PbTx-2 (i.e., representing an average of 1.8 pg of PbTx-2 exuded by each live *K. brevis* cell).

Experiment 2 tested whether *K. brevis* exudates reduce competitor photosynthetic efficiency as a potential mechanism for allelopathy. For each of the same five competitor species used in experiment 1, 5–6 pairs of 10-mL culture tubes containing 2.8 mL of L1 + silicate media were inoculated with 200 μL of phytoplankton culture. When the competitor species reached exponential growth stage, bloom or nonbloom extract (from experiment 1, generated from 3.0 mL of field sample) was dissolved in 5.0 μL of DMSO and added to each tube. All tubes were incubated in the dark for 1 h. For each species, we determined photosynthetic efficiency of photosystem II, measured as $F_v : F_m$, where $F_v = F_m - F_0$ (e.g., Parkhill et al. 2001; Miller-Morey and Van Dolah 2004). F_0 (initial fluorescence) was determined using a Turner Designs TD-700 fluorometer. F_m (maximal fluorescence) was determined by

adding 9.6 μL of 10 mol μL^{-1} aqueous 3′-(3,4-dichlorophenyl)-1′,1′-dimethylurea, waiting 30 s, and measuring the fluorescence.

To determine the competitor growth stage at which photosynthetic efficiency is most inhibited, we repeated this experiment using extracts of *K. brevis* cultures, adding extracts during competitor lag, early exponential growth, and stationary growth stages. Sixty culture tubes for each species were prepared by inoculating 2.8 mL of L1 + silicate media with 200 μL of phytoplankton culture. For each species, 20 tubes were removed from the pool at each of three separate growth stages; the remaining tubes were allowed to grow until reaching later growth stages. *K. brevis* extract (combined allelopathic extract used in experiment 1, generated from 3.0 mL of culture) dissolved in 5.0 μL of DMSO was added to each of 10 tubes and 5.0 μL of DMSO (control) was added to the other 10 tubes. Preliminary experiments indicated no significant difference between solvent controls and media extract controls for any of the species considered (data not shown; $p = 0.31$–1.00; Wilcoxon sign–rank test). Five pairs of tubes were then incubated in the dark for 1 h, and $F_v : F_m$ was determined as described above. The other five pairs were allowed to grow for 23 h and then incubated in the dark for 1 h before $F_v : F_m$ was determined. This process was repeated with competitor cultures in early exponential growth and stationary stages.

Because enzyme activity has been reported to be a target of allelopathy (Windust et al. 1996; Sukenik et al. 2002;) and active esterases are indicative of healthy, functional cells (Agusti et al. 1998; Agusti and Sanchez 2002), experiment 3 assessed whether *K. brevis* exudates inhibit competitor esterase activity as a potential mechanism for allelopathy, using the fluorescent dye fluorescein diacetate (FDA) (Agusti and Sanchez 2002). This dye passes freely through the membrane of phytoplankton cells; once inside, cells with active esterases hydrolyze the ester bonds of FDA. The hydrolyzed dye is fluorescent, and cannot exit the cell. Thus, cells with active esterases fluoresce, and cells without active esterases do not. For each competitor species, 10-mL tubes containing 2.0 mL of phytoplankton culture were incubated at 22°C with *K. brevis* extract (from experiment 1, combined allelopathic extract generated from 2.0 mL of *K. brevis* culture) in 3.3 μL of DMSO (or 3.3 μL of DMSO for control tubes) ($n = 7$). Preliminary experiments indicated no differences in the esterase activity of cultures exposed to DMSO or extract of L1 + silicate media in DMSO (data not shown; $p = 0.50$–1.00; Wilcoxon sign–rank test). After 2 h, 500 μL of each culture was stained with 2.5 μL of 25 mol μL^{-1} FDA in DMSO (to assess the number of cells with active esterases) and 500 μL of each culture was stained with Lugol solution (to determine the total cell concentration). Samples stained with FDA were kept in the dark for 10–30 min before excitation at 451 nm. FDA-stained cells emitting light at 510 nm were counted using fluorescence microscopy on an Olympus IX-50 inverted microscope with a Palmer-Maloney settling chamber. Samples preserved with Lugol solution were counted with light microscopy using the same instrument. We assessed the proportion of cells with active esterases by dividing the number of cells stained with FDA by the number of cells stained with Lugol solution for each sample.

Experiment 4 determined the effect of *K. brevis* allelopathic compounds on competitor cell membrane permeability using the fluorescent dye SYTOX green (Brussaard et al. 2001). This dye binds to cellular nucleic acids but cannot permeate healthy membranes; thus only cells with damaged membranes fluoresce. The combined allelopathic *K. brevis* culture extract from experiment 1 was added to each competitor species as described for experiment 3 ($n = 7$–8). Preliminary experiments indicated no differences in membrane permeability from cultures exposed to DMSO or extract of L1 + silicate media in DMSO (data not shown; $p = 0.25$–1.00; Wilcoxon sign–rank test). After 2 h, 500 μL of each culture was stained with 5.0 μL of 0.50 mol μL^{-1} SYTOX green in DMSO (to assess the number of cells with permeable membranes) and 500 μL was stained with Lugol solution (to determine the total cell concentration). Samples stained with SYTOX green were kept in the dark for 10–30 min before excitation at 504 nm. SYTOX green-stained cells emitted light at 523 nm. Cells stained with SYTOX green and Lugol solution were counted and the proportion of cells with permeable membranes was assessed as in experiment 3. In addition to the total proportion of cells with permeable membranes, we also assessed the fraction of stained cells that were motile (actively swimming) for the two dinoflagellates (*P. minimum* and *Akashiwo* cf. *sanguinea*). Motility could not be assessed by microscopy for the three diatom species.

Experiment 5 tested whether *K. brevis* exudates strongly chelate iron, potentially limiting the access of other phytoplankton species to this essential nutrient. We analyzed *K. brevis* extracellular extracts for the presence of siderophores, compounds that bind to iron, using a universal chemical assay, in which siderophores compete with chrome azurol S (CAS) for Fe(III) (Schwyn and Neilands 1987). When bound to iron, CAS absorbs strongly at 630 nm. A decrease in absorption at this wavelength indicates that iron is bound to a siderophore. Made according to Schwyn and Neilands (1987), 1.5 mL of the CAS shuttle solution was combined with extract of *K. brevis* generated from 3.0 mL of culture dissolved in 1.5 mL of deionized (DI) water, extract of *K. brevis* generated from 30 mL of culture (i.e., 10× natural concentration) dissolved in 1.5 mL of DI water, or extract of L1 + silicate generated from either 3.0 mL or 30 mL of media (control) prepared the same way ($n = 3$ each). All samples were equilibrated for 4.5 h, after which the absorbance of each sample was measured at 630 nm using a Spectronic 21D spectrophotometer. This experiment was performed both for the allelopathic combined extract of *K. brevis* culture (used in experiment 1) and nonallelopathic extract of *K. brevis* (from a nonallelopathic culture generated in experiment 1; 1.52×10^4 cells mL^{-1}, containing 17 ng mL^{-1} PbTx-2, representing an average of 1.1 pg of PbTx-2 exuded by each *K. brevis* cell). The relative abundance of siderophores normalized to media controls was calculated using Equation 3: % siderophore

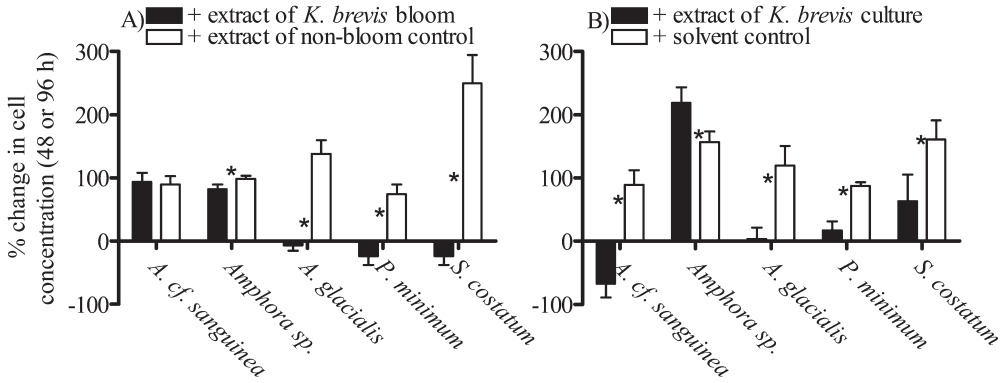

Fig. 1. Effects of extracellular extracts of (A) *K. brevis* bloom or (B) cultures on the growth of five competing phytoplankton species (experiment 1). Changes in cell concentration were assessed by in vivo fluorescence after 48 h for all species except the slow-growing *Akashiwo* cf. *sanguinea*, whose final concentration was measured after 96 h. Error bars in this and subsequent figures indicate 1 SD. Significance is marked with an asterisk (*) ($p \leq 0.05$; $n = 5$–10).

$$\text{bound iron} = \frac{A(\text{media}) - A(K.\,brevis)}{A(\text{media})} \times 100\% \text{ where}$$

A(media) is the absorbance of media extracts combined with the CAS shuttle solution and A(*K. brevis*) is the absorbance of *K. brevis* extracts combined with the CAS shuttle solution.

Statistical analyses—Preliminary analysis using the Kolmogorov–Smirnov statistical test determined that the percentage growth of all phytoplankton species used in our experiments was normally distributed (Zar 1999). Therefore, experiment 1 was analyzed using parametric tests. However, we were unable to perform similar analyses for the data acquired in experiments 2–5 because sample sizes were too small; therefore these experiments were analyzed using nonparametric tests.

To analyze the experiment 1 data testing allelopathic effects of *K. brevis* bloom and culture extracts, the percentage change in fluorescence was compared for treatments versus controls of each species using a paired two-tailed *t*-test (Graphpad Prism 4.0). To analyze data testing allelopathic effects of several extracts of *K. brevis* cultures assayed concurrently, the percentage growth of *Asterionellopsis glacialis* after 48 h was compared with controls using a one-way analysis of variance with Tukey post hoc test (SYSTAT 9). Differences were accepted as significant when $p \leq 0.05$.

For experiment 2, treatments and controls were compared by two methods (Graphpad Prism 4.0). We used a one-tailed Wilcoxon sign–rank test to test whether the photosynthetic efficiency of treatments was lower than that of controls ($p \leq 0.05$ deemed significant). However, nonparametric tests are less powerful than their parametric counterparts, so no adjustment could be made for multiple comparisons without losing the ability to detect significant differences. To limit the possibility that we committed a type I error, we also analyzed data using a paired two-tailed *t*-test with a Bonferroni adjustment (thus, $p \leq 0.017$ was deemed significant).

Experiments 3 and 4 were analyzed using a two-tailed Wilcoxon sign–rank test (Graphpad Prism 4.0). We compared the effect of *K. brevis* extracts versus controls for each species, for which differences were deemed significant when $p \leq 0.05$. Because several comparisons were made for each species, statistical tests comparing the proportion of stained cells that were motile between treatments and controls were analyzed using a Wilcoxon sign–rank test (Graphpad Prism 4.0) with a Bonferroni adjustment for multiple comparisons such that differences were accepted as significant when $p \leq 0.017$.

Experiment 5 was analyzed by comparing results of siderophore activity tests for an allelopathic versus non-allelopathic *K. brevis* extract with a Mann–Whitney *U*-test. Differences were accepted as significant when $p \leq 0.05$.

Results

Bloom composition—Approximately 80% of the cells in the three field samples collected from a single Florida bloom in 2006 were *Karenia* species. However, we estimate that low concentrations of at least 15 other species were also present. The most abundant included *Oxyphysis oxytoxoides* and *Skeletonema costatum*, as well as species from the *Chaetoceros*, *Baceriastrum*, and *Scrippsiella* genera. Several unidentified dinoflagellates and pennate diatoms were also present. The composition of the nonbloom sample collected on the same day was quite different, containing only a few of the species also present in bloom samples. More than half of cells present in the nonbloom sample were pennate diatoms, including *Nitzchia longissima*. One dinoflagellate, *Prorocentrum mexicanum*, made a significant contribution to the nonbloom community. Several other species, including the dinoflagellate *Ceratium furca* and a small number of unidentified centric diatoms, were also present.

Allelopathic effects of extracellular extracts from K. brevis *bloom samples and cultures*—Compounds exuded by a *K. brevis* bloom inhibited the growth of four of five competitor species when compared with effects of extracts of nonbloom seawater, but the degree of inhibition varied among species (Fig. 1A). Three competitor species, *Asterionellopsis glacialis*, *Prorocentrum minimum*, and *S. costa-*

tum, were strongly inhibited by the *K. brevis* bloom extract, such that their cell concentrations were lower after 48–96 h than at the start of the experiment when they were in exponential growth stage ($p \leq 0.001$ for all). The growth of one competitor, *Amphora* sp., was inhibited by 16% ± 9% ($p = 0.007$), and another species, *Akashiwo* cf. *sanguinea*, was not inhibited ($p = 0.40$).

Seven of 16 *K. brevis* cultures were allelopathic to *A. glacialis*. Five of these were strongly allelopathic, suppressing *A. glacialis* growth by ≥90% in 48 h ($p \leq 0.001$), and two were moderately allelopathic (22–41% suppression; $p \leq 0.05$). Nine extracts did not suppress *A. glacialis* growth at all ($p = 0.13$–0.94; data not shown). The five most allelopathic extracts were combined, and found to adversely affect four of five competitor species tested (Fig. 1B). This combined extract killed one species, *Akashiwo* cf. *sanguinea*, and reduced the growth of three species by 60–97%: *Asterionellopsis glacialis*, *P. minimum*, and *S. costatum* (all $p < 0.001$). One species, *Amphora* sp., was stimulated by *K. brevis* extracts ($p < 0.001$).

Reduction of competitor photosynthetic efficiency by K. brevis *allelopathy*—All five competitor species experienced decreased photosynthetic efficiency within 1 h of exposure to *K. brevis* bloom extract (Fig. 2A). *S. costatum* was intensely affected, with a 68% reduction in PSII efficiency. The photosynthetic efficiencies of *Akashiwo* cf. *sanguinea, Amphora* sp., *Asterionellopsis glacialis*, and *P. minimum* were inhibited by 19–43% (Wilcoxon sign–rank test: $p \leq 0.031$ for all; *t*-test: $p \leq 0.016$ for all).

Consistent with effects of the bloom extract, extracellular extracts of cultured *K. brevis* inhibited the photosynthetic efficiencies of all five competitor species during at least one stage of their growth (Fig. 2B). *Asterionellopsis glacialis, P. minimum*, and *S. costatum* PSII was inhibited during their lag, exponential growth, and stationary stages ($p = 0.031$ for each species, Wilcoxon sign–rank test; $p \leq 0.01$ for each species, *t*-test). The photosynthetic efficiency of *S. costatum* was consistently strongly suppressed by 65–77%, whereas *A. glacialis* and *P. minimum* were most inhibited during lag stage (49–67%), rather than in exponential growth (39–48%) or stationary stage (20%). *Akashiwo* cf. *sanguinea's* photosynthetic efficiency was lowered by 33–53% by *K. brevis* extracts generated in lag and exponential growth stage ($p = 0.031$ in both cases, Wilcoxon sign–rank test; $p < 0.01$, *t*-test) but not in stationary stage ($p = 0.41$, Wilcoxon sign–rank test). Interestingly, *Amphora* sp., whose growth over 48 h was not suppressed by cultures of *K. brevis* (Fig. 1B), suffered a 65% reduction of photosynthetic efficiency when exposed to *K. brevis* extracts for 1 h in lag phase ($p = 0.031$, Wilcoxon sign–rank test; $p < 0.001$, *t*-test), but photosynthetic efficiency was unaffected if *K. brevis* extracts were added at later growth stages (exponential growth stage, $p = 0.16$; stationary stage, $p = 0.31$; Wilcoxon sign–rank test) (Fig. 2B). Additional measurements of photosynthetic efficiency generated 24 h after the addition of extract were similar for all species except *A.* cf. *sanguinea* and *S. costatum*, which showed even more dramatically reduced photosynthetic efficiency after 24 h (data not shown) than after 1 h (Fig. 2B).

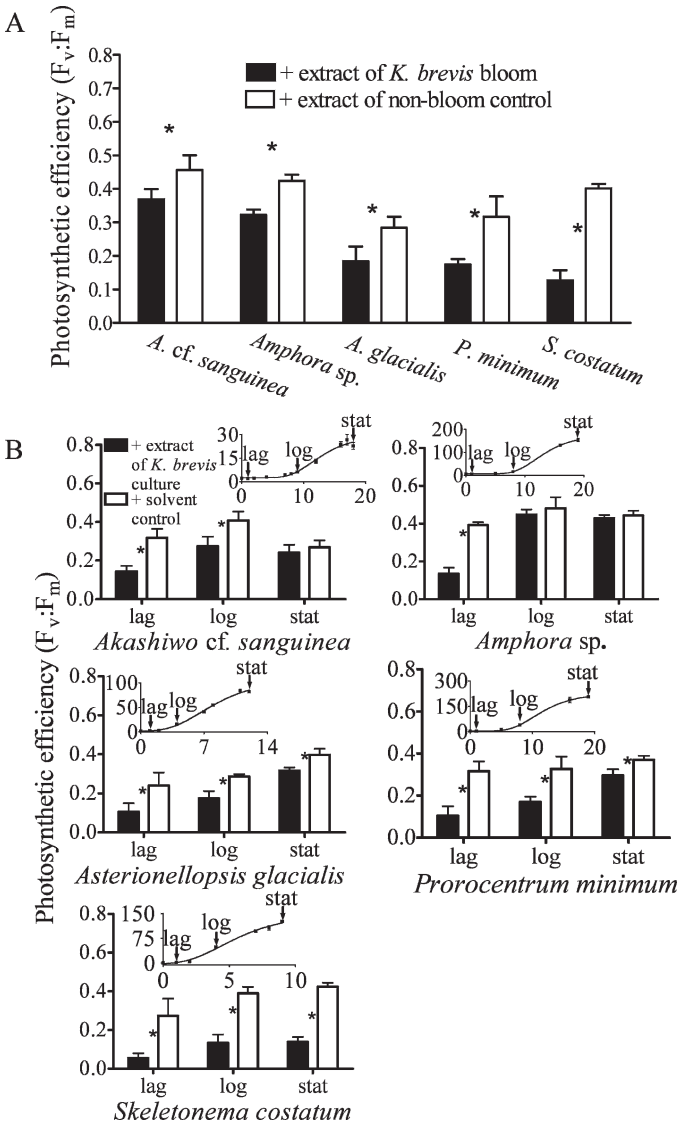

Fig. 2. Effects of *K. brevis* allelopathy on competitor photosynthetic efficiency (experiment 2). (A) During exponential growth stage, competitor species were treated with extracellular extracts of either *K. brevis* bloom or nonbloom sample. (B) Competitor species were treated with an allelopathic extracellular extract of cultured *K. brevis* or a solvent control at three different competitor growth stages (lag, exponential growth [log], or stationary [stat] phases). Insets show growth curve of competitors, where number of days is on the *x*-axis, and fluorescence (μg L^{-1} Chl *a*) is on the *y*-axis. Arrows indicate the place in the growth curve where competitors were harvested for this experiment. Significance is marked with an asterisk (*) ($p \leq 0.017$, *t*-test; $p \leq 0.05$, Wilcoxon sign–rank test; $n = 5$).

Effects of Karenia brevis *allelopathy on competitor esterase activity—K. brevis* extracellular extracts had no effect on the esterase activity of any competitor species tested (Fig. 3). For each species, no significant difference was found in the proportion of competitor cells with active esterases for those exposed to cultured *K. brevis* extract versus those exposed to a solvent control ($p = 0.11$–0.81, Wilcoxon sign–rank test).

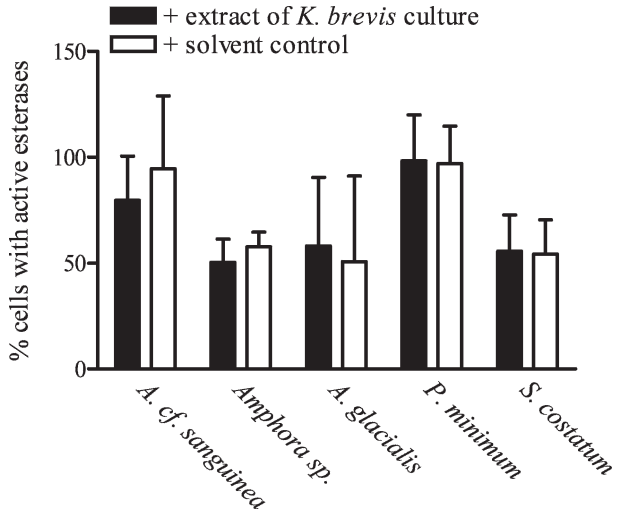

Fig. 3. Allelopathic effect of cultured *K. brevis* extracellular extract on the esterase activity of five competing phytoplankton species measured with fluorescein diacetate (FDA) (experiment 3). For all five species, contrasts between treatments and controls yielded $p > 0.10$ ($n = 7$).

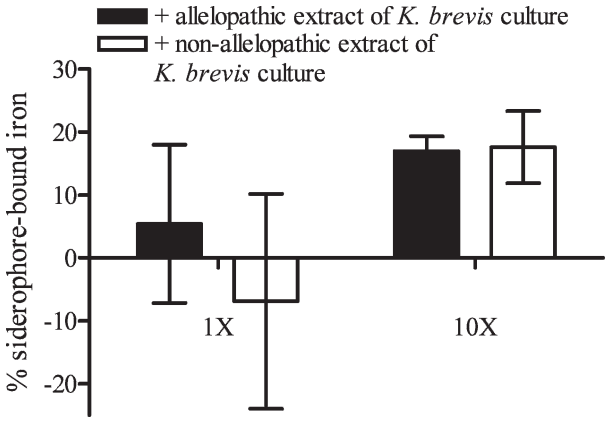

Fig. 5. Test of siderophore activity for cultured *K. brevis* extracellular extracts (experiment 5). The presence of iron-binding compounds in *K. brevis* extracellular extracts was tested at natural (1×) and 10 times natural (10×) concentration using a chrome azurol S (CAS) shuttle solution (Schwyn and Neilands 1987). Data depict the proportion of siderophore-bound iron relative to the appropriate media control. Contrasts between allelopathic and nonallelopathic extract treatments yielded $p \geq 0.40$ at both concentrations ($n = 3$).

Effects of K. brevis *allelopathy on competitor membrane permeability*—Exposing competitors to cultured *K. brevis* extracellular extracts increased the proportion of cells with permeable (damaged) membranes for three species: *Akashiwo* cf. *sanguinea* ($p = 0.016$; Wilcoxon sign–rank test), *Asterionellopsis glacialis* ($p = 0.016$), and *P. minimum* ($p = 0.008$) (Fig. 4A). The effects on membrane permeability of two species, *S. costatum* and *Amphora* sp., were insignificant ($p = 0.47$ and 0.38, respectively). For *Akashiwo* cf. *sanguinea* and *P. minimum*, whose motility could be directly observed by light microscopy, 2-h exposure to allelopathic extracts increased the proportion of motile cells with damaged membranes ($p = 0.016$ and 0.008, respectively) (Fig. 4B). The proportion of membrane-permeable nonmotile *A.* cf. *sanguinea* cells was not significantly different between cells exposed to extracts versus those exposed to a solvent control ($p = 0.31$), and was only marginally significant for *P. minimum* ($p = 0.042$).

Siderophore activity of K. brevis *extracts*—No significant differences were detected in iron-chelating activity between *K. brevis* allelopathic and nonallelopathic extracts (Fig. 5), regardless of the concentration at which the extracts were tested ($p = 0.40$ for 1× natural concentration and $p = 0.70$ for 10× natural concentration of cultured *K. brevis* extract).

Discussion

Karenia brevis *blooms are allelopathic*—The growth of three Gulf of Mexico phytoplankton species was strongly inhibited by compounds exuded during blooms of the red tide dinoflagellate *K. brevis*, and the growth of one species

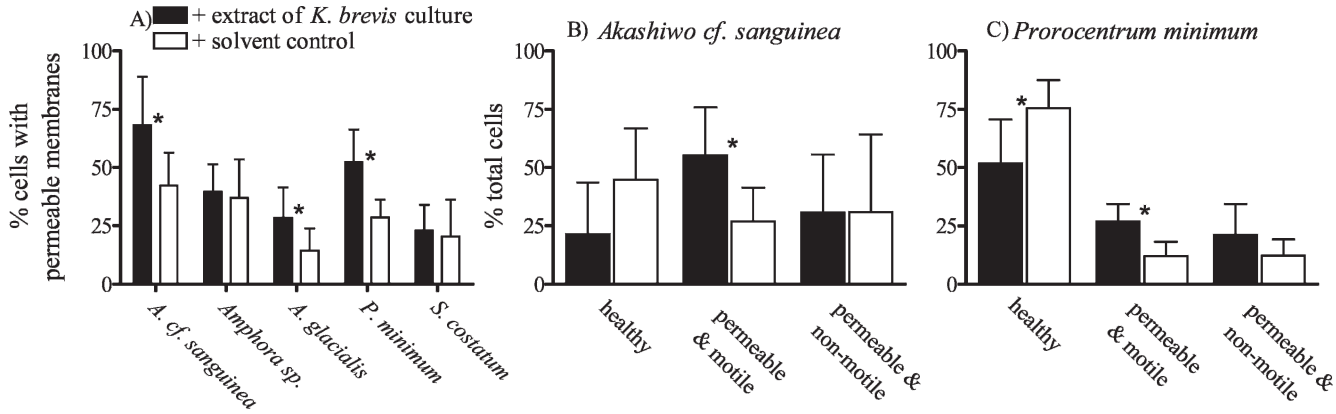

Fig. 4. Allelopathic effect of cultured *K. brevis* extracellular extract on the membrane permeability of five competing phytoplankton species using SYTOX green to assess damaged (permeable) cells (experiment 4). (A) Cells with damaged membranes for all species tested ($p \leq 0.05$; $n = 7$–8). (B) *Akashiwo* cf. *sanguinea* and (C) *Prorocentrum minimum.* Motile (swimming) cells with damaged membranes ($p \leq 0.017$; $n = 8$). Significant difference between treatments and controls is marked with an asterisk (*).

was very slightly inhibited, providing strong evidence that *K. brevis* is allelopathic in the field (Fig. 1A). We found that cultures of *K. brevis* also inhibited the growth of competitor species (Fig. 1B), consistent with previous studies using cultured strains of *K. brevis* (Freeburg et al. 1979; Kubanek et al. 2005). Thus, allelopathy may play a role in enabling *K. brevis* to outcompete other phytoplankton species in the field and to dominate the plankton community during blooms.

Three competitor species that strongly inhibited *K. brevis* cultures were at least as inhibited by compounds exuded by *K. brevis* blooms (Fig. 1), even though *K. brevis* cell concentrations in cultures were almost 100 times greater than in natural bloom samples. Waterborne concentrations of PbTx-2, the major brevetoxin present in bloom waters (Pierce et al. 2005), were nearly 20 times higher in *K. brevis* cultures than in field samples, supporting the previous finding that brevetoxins are unlikely to be responsible for most cases of *K. brevis* allelopathy (Kubanek et al. 2005).

The composition of the plankton community is likely to play a role in the allelopathic potency of *K. brevis* blooms. Approximately 20% of cells in bloom samples belonged to non-*Karenia* taxa, and the representation of these community members differed between bloom and nonbloom samples collected on the same day (*see* Results). Therefore, it is possible that species other than *K. brevis* contributed to bloom allelopathy, possibly accounting for the observation that growth of *Amphora* sp. was modestly suppressed by bloom but not cultured *K. brevis* exudates (Fig. 1). However, since none of the non-*Karenia* species made up more than 5% of the bloom community, it is difficult to predict which other species may be involved in bloom allelopathy. Because allelopathic effects of cultured *K. brevis* and bloom samples were similar for three of five species tested (*Asterionellopsis glacialis, P. minimum, S. costatum*; Fig. 1), it appears likely that *K. brevis* is responsible for most of the allelopathic potency of bloom samples.

It is not clear why one competitor, *Akashiwo* cf. *sanguinea*, was inhibited by *K. brevis* cultures but not by waterborne compounds from *K. brevis* blooms (Fig. 1). It is possible that *A.* cf. *sanguinea* is less sensitive to *K. brevis* allelopathy than other species, and that the 2006 bloom we sampled of *K. brevis* was not dense enough to inhibit this competitor's growth. Kubanek et al. (2005) reported that *A*. cf. *sanguinea* was not inhibited by filtrates of cultured *K. brevis*. However, in that case, filtrates were tested at lower concentrations than extracellular extracts used in this study, supporting the hypothesis that *A*. cf. *sanguinea* may be suppressed only by compounds from high concentrations of *K. brevis* cells. It is also possible that *A.* cf. *sanguinea* was not susceptible to the *K. brevis* strains present in the 2006 bloom. Our laboratory cultures of *K. brevis* consisted of a single strain, whereas the field population was likely more diverse. No studies have addressed the genetic diversity of *K. brevis* blooms, but phytoplankton blooms are often genetically complex (e.g., John et al. 2004; Wilson et al. 2005). *A.* cf. *sanguinea* may be unaffected by bloom extracts because the allelopathic potency of *K. brevis* varies among strains, possibly in a species-specific manner. *K. brevis* also may produce compounds allelopathic to *A.* cf. *sanguinea* only under certain conditions that may have been absent during field sampling (e.g., under a particular nutrient regime or in the presence of particular plankton community members).

Production of allelopathic compounds and their effects on competitors are variable—Both the susceptibility of competitor species to allelopathy and the production of allelopathic compounds by *K. brevis* were variable. Of the 16 extracts generated from exponential growth stage cultures of a single *K. brevis* strain, seven were allelopathic to the diatom *Asterionellopsis glacialis* (*see* Results). Allelopathic effects also varied for natural *K. brevis* bloom samples; allelopathic effects of 10 bloom samples collected during two separate years ranged from complete suppression of competitor growth to no suppression at all, although the pooled data indicate that *K. brevis* blooms are significantly allelopathic (Fig. 1A; Prince et al. unpubl. data).

Variability of allelopathic effect among cultures may be influenced by nutrient limitation, associated bacteria, and lack of selection pressure, as well as other factors such as growth stage and pH (Schmidt and Hansen 2001). Previous research on *K. brevis* indicated that allelopathic effects of cultures varied among strains and growth stages (Kubanek et al. 2005). Although attempts were made to consistently control growth conditions, it is likely that *K. brevis* cultures had small differences in pH, were extracted at slightly different growth stages and cell densities, were inoculated with cultures at different growth stages, or had accumulated mutations over time because of lack of selective pressure. Thus, we are currently unaware of which factors control the production of allelopathic compounds by *K. brevis*, although this issue begs further investigation.

Even when allelopathic compounds were produced by *K. brevis*, they did not affect all species to the same extent (Fig. 1). *Akashiwo* cf. *sanguinea* was more susceptible to *K. brevis* culture than bloom exudates, whereas *Amphora* sp. was slightly suppressed by bloom exudates but actually stimulated by *K. brevis* culture exudates, suggesting that these two competitor species are affected by different *K. brevis* compounds. Our finding of the species-specific nature of allelopathy is consistent with results from previous studies involving *K. brevis* (Freeburg et al. 1979; Kubanek et al. 2005) and other bloom-forming dinoflagellates (Arzul et al. 1999; Sugg and Van Dolah 1999). Kubanek et al. (2005) and Freeburg (1979) found that compounds produced by *K. brevis* inhibited the growth of the diatoms *Asterionellopsis glacialis* and *S. costatum*, species that we also report to be susceptible to *K. brevis* allelopathy. In general, however, we saw stronger allelopathic effects than Kubanek et al. (2005), probably because of the enhanced efficiency and avoidance of inorganic nutrient artifacts of the extracellular extraction method used in the current study.

The observed variability of *K. brevis* allelopathy may have implications for bloom dynamics. *K. brevis* blooms typically occur in four general stages: initiation, growth, maintenance, and termination (Steidinger et al. 1998).

Because our *K. brevis* extracts came from exponential growth stage cultures with high cell concentrations reminiscent of a dense bloom, our data (Fig. 1B) support the hypothesis that *K. brevis* uses allelopathy to maintain blooms, but we cannot assess whether allelopathy is important during bloom initiation. Extracts of a *K. brevis* bloom sampled during maintenance stage were strongly allelopathic (Fig. 1A), further supporting the idea that allelopathy may allow *K. brevis* to maintain blooms. Future studies should consider the allelopathic activity of *K. brevis* blooms at different cell concentrations and bloom stages.

Karenia brevis *lowers photosynthetic efficiency of competitors*—Compounds exuded by *K. brevis* inhibited photosynthesis of competing phytoplankton, suggesting a potential mechanism for allelopathy (Fig. 2). The maximum quantum yield of PSII is frequently used as a proxy for photosynthetic efficiency and consequently as a general measure of cell stress (e.g., Parkhill et al. 2001; Miller-Morey and Van Dolah 2004); a decrease in PSII efficiency can result from a variety of factors including nutrient limitation (Parkhill et al. 2001), shading (Villareal and Morton 2002), metal toxicity (Miller-Morey and Van Dolah 2004), and allelopathy (Figueredo et al. 2007). However, several studies have warned about the limitations of this method, claiming that nutrient limitation cannot be detected with balanced growth conditions (Parkhill et al. 2001), that a decrease in maximal quantum yield is specific to some stressors but others (e.g., heat shock) cannot be detected (Miller-Morey and Van Dolah 2004), and that nutrient stress may be missed because of the time required for a fluorescence response to nutrient starvation (Singler and Villareal 2005). These studies indicate that results of experiments measuring photosynthetic efficiency should be interpreted with care, and a lack of inhibition of PSII may not indicate that cells are not stressed. However, in our experiments, compounds exuded by *K. brevis* inhibited the PSII of competitors within 1 h, strongly indicating competitor stress (Fig. 2).

Inhibition of competitor photosynthesis by *K. brevis* extracellular extracts appeared to parallel the negative effects on competitor growth (Figs. 1, 2). Species whose growth was strongly suppressed by bloom extracts (*Asterionellopsis glacialis, P. minimum*, and *S. costatum*) also experienced substantial reduction in photosynthetic efficiency (Figs. 1A, 2A). Parallels between reduced photosynthesis and growth inhibition were not as strong when competitor species were exposed to *K. brevis* culture extracts. However, the species least sensitive to general allelopathic effects, *Amphora* sp., appeared most resistant to PSII inhibition (Figs. 1B, 2B). The efficiency of PSII measured after 1-h exposure to allelopathic extracts may be a more sensitive indicator of competitor stress than effects on growth measured over 2–4 d, since *K. brevis* bloom extracts slightly inhibited PSII of *Akashiwo* cf. *sanguinea* but did not significantly suppress *A.* cf. *sanguinea* growth (Figs. 1A, 2A). Because of the sensitivity and speed of this assay, decreases in PSII efficiency may also provide information about when competitors are most susceptible to allelopathy. We found that *K. brevis* compounds most strongly suppressed competitor photosynthetic efficiency when competitor cell concentrations were low (i.e., lag stage before exponential growth) (Fig. 2B), although we did not determine if this effect is due to low cell concentrations or increased physiological susceptibility during lag stage. Thus, one way *K. brevis* may be able to maintain nearly monospecific blooms is by preventing competitors from reaching high cell densities.

Inhibition of PSII is commonly proposed as a mechanism for allelopathy; however, it is difficult to determine whether reduced efficiency of PSII is a direct target or a symptom of allelopathy-induced stress. Both bloom and culture filtrates of the cyanobacterium *Cylindrospermopsis raciborskii* inhibited the photosynthetic efficiency of competing cyanobacteria (Figueredo et al. 2007). Other studies have further elucidated the mechanism by which photosynthetic efficiency is inhibited. Von Elert and Juttner (1997) showed that while photosystem I of a cyanobacterial competitor was unaffected by compounds produced by the cyanobacterium *T. doliolum*, overall oxygen evolution was reduced because of inhibited electron flow in PSII. In addition, Srivastava et al. (1998) found that when the cyanobacterium *Anabaena* P9 was exposed to fishcherellin A, electron transport in PSII decreased, followed by inactivation of reactive centers, then grouped units within PSII were separated. However, neither our nor any of these studies have yet shown that PSII is the primary target of allelopathy rather than a secondary effect of another target.

K. brevis *disrupts competitor membrane integrity but not cellular esterase activity*—Using the fluorescent stain SYTOX green, we found that allelopathic compounds exuded by *K. brevis* increased the membrane permeability of three competitor species (Fig. 4). SYTOX green is usually used to distinguish between live and dead cells (Brussaard et al. 2001), but we found that many dinoflagellate cells with membranes permeable to this dye were swimming and therefore certainly not dead after 2-h exposure to *K. brevis* extracts (Fig. 4B). In fact, *K. brevis* exudates primarily increased the proportion of *motile* cells with damaged membranes, indicating that allelopathic compounds increased membrane permeability *before* competitor cells died or ceased swimming. This suggests that membrane damage is not a side effect of cell death, but rather a likely target for allelopathic compounds. We therefore propose that *K. brevis* may outcompete other phytoplankton by producing allelopathic compounds that damage the cell membranes of competitors.

We did not detect significant differences in esterase activity for competitor species exposed to allelopathic compounds versus controls, using the fluorescent stain FDA (Fig. 3), suggesting that compounds produced by *K. brevis* do not initially target esterases as a mechanism for allelopathy. In other systems, esterase activity is used as a proxy for ''metabolic vigor'' (Dorsey et al. 1989), and has been shown to decrease because of viral infection (Lawrence et al. 2006), nutrient limitation (Brookes et al. 2000), and metal toxicity (Franklin et al. 2001). Although no previous studies have investigated whether esterase activity is a target for allelopathic compounds, other enzymes can

be affected, including phosphatases (Windust et al. 1996) and carbonic anhydrase (Sukenik et al. 2002). Thus, establishing that *K. brevis* did not target the esterases of competitors ruled out a potentially important allelopathic pathway.

K. brevis *allelopathy is not mediated by removal of waterborne iron*—Iron availability is an important factor governing phytoplankton abundance, especially in high-nutrient low-chlorophyll areas in the ocean (Hutchins et al. 1999) and may play a role in triggering harmful algal blooms (Wells et al. 1991). Several species of the bloom-forming diatom genus *Pseudonitzchia* have been shown to increase toxin production in response to iron limitation (Wells et al. 2005), indicating that harmful algal bloom species may exude compounds to compete for iron. More than 99% of iron in oceanic waters is chelated by organic ligands (Rue and Bruland 1995), some of which are siderophores, iron-binding compounds produced by marine bacteria and cyanobacteria and utilized by eukaryotic phytoplankton in response to iron limitation (Wilhelm and Trick 1994; Hutchins et al. 1999). We hypothesized that *K. brevis* cultures may be allelopathic because *K. brevis* or its symbiotic bacteria produce iron-binding compounds that competitors were unable to access. However, we found no differences in iron-chelating activity between allelopathic and nonallelopathic *K. brevis* extracellular extracts (Fig. 5), demonstrating that removal of iron from cultures could not be the mechanism *K. brevis* uses to suppress competitor growth.

Overall, we found that *K. brevis* blooms were allelopathic to four of five competing phytoplankton tested, and that *K. brevis* cultures exuded compounds with similar inhibitory effects. Competitor species whose growth was most inhibited by *K. brevis* bloom exudates also suffered substantial decreases in photosynthetic efficiency. In addition, compounds exuded by *K. brevis* damaged cellular membranes of three competitor species, indicating a potential mechanism for *K. brevis* allelopathy. Our results suggest that *K. brevis* exudes allelopathic compounds that may facilitate its occasional dominance of the phytoplankton community, potentially leading to red tides.

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