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Are offshore phytoplankton susceptible to *Karenia brevis* allelopathy?

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The bloom-forming dinoflagellate *Karenia brevis* produces a suite of allelopathic compounds that inhibit the growth of several phytoplankton competitors in laboratory experiments. However, it is less clear how allelopathy affects competition in the field, including whether allelopathic compounds impact *K. brevis* bloom dynamics. We investigated the extent to which phytoplankton species typically found offshore in the Gulf of Mexico, where *K. brevis* blooms initiate, are sensitive to *K. brevis* allelopathy. Natural assemblages of offshore phytoplankton dominated by diatoms were largely resistant to *K. brevis* allelopathy, even experiencing slight stimulation of growth from exposure to *K. brevis* exudates. When tested in pair-wise laboratory experiments, four diatom species often found offshore in the Gulf of Mexico exhibited varying degrees of resistance to *K. brevis* similar to that observed with field assemblages, although some competitors displayed a trend toward growth inhibition. In concurrent pair-wise laboratory experiments, four diatom species whose nearshore habitats frequently experience dense *K. brevis* blooms also demonstrated a range of responses to *K. brevis* allelopathy, from strong sensitivity to resistance. Overall, Gulf of Mexico phytoplankton that co-occur with *K. brevis* blooms in both nearshore and offshore environments respond variably to *K. brevis* allelopathy.

KEYWORDS: allelopathy; *Karenia brevis*; competition; chemical ecology; harmful algal bloom

INTRODUCTION

Karenia brevis is a red tide dinoflagellate that blooms almost annually in the Gulf of Mexico (Tester and Steidinger, 1997), and produces brevetoxins (Baden, 1989), neurotoxins responsible for fish and marine mammal mortality events (Flewelling *et al.*, 2005). Brevetoxins also cause neurotoxic shellfish poisoning in humans (Landsberg *et al.*, 2009). Allelopathy, the production and release of compounds to inhibit competitors, may be one mechanism that *K. brevis* uses to maintain large, nearly monospecific blooms in the Gulf of Mexico (Kubanek *et al.*, 2005). Although *K. brevis* is infamous for

its toxicity toward vertebrates, its allelopathic effects are attributed to a separate suite of unstable compounds, distinct from brevetoxins, which inhibit the growth of other phytoplankton (Poulson *et al.*, 2010; Prince *et al.*, 2010).

Researchers have questioned whether allelopathy can function as a viable competitive mechanism in the plankton, often citing the rapid dilution of allelopathic compounds as a major drawback to this competitive strategy (Lewis, 1986; Flynn, 2008). It seems unlikely that allelopathy facilitates bloom formation since in early bloom stages, concentrations of algal cells are typically too low to release substantial amounts of allelopathic compounds (Jonsson *et al.*, 2009). In contrast, phytoplankton in the vicinity of large, established blooms are more likely to be exposed to high levels of allelopathic compounds. At high cell concentrations associated with bloom maintenance, allelopathic compounds may accumulate in the water column, presenting sensitive competitors with an inhospitable environment for growth and potentially selecting for resistance traits among competitors.

Karenia brevis blooms are impacted by physical forces that concentrate blooms from offshore initiation sites, moving *K. brevis* populations nearshore via wind-driven currents (Walsh *et al.*, 2006; Hetland and Campbell, 2007). In the eastern Gulf of Mexico, blooms often form 18 to 74 km offshore on the West Florida Shelf (Steidinger and Haddad, 1981), accumulating along shelf fronts and moving inshore as blooms progress (Tester and Steidinger, 1997). Nearshore, very dense blooms ($>1\,000\,000$ cell L^{-1}) can persist for months (Vargo, 2009) where the environment is greatly influenced by land runoff that supplies nearshore blooms with nutrients (Brand and Compton, 2007). Dense nearshore blooms of *K. brevis* frequently encounter competing phytoplankton including the diatoms *Asterionellopsis glacialis* and *Skeletonema* spp. (Turner and Hopkins, 1974; Badylak *et al.*, 2007), both of which have been shown to be sensitive to *K. brevis* allelopathy (Prince *et al.*, 2008; Poulson *et al.*, 2010). Previous studies investigating *K. brevis* allelopathy have typically focused on such nearshore competitors to *K. brevis* and have not investigated how plankton common in offshore environments respond to *K. brevis*.

Because blooms of *K. brevis* initiate on the West Florida Shelf, our objective was to determine if competitors present farther offshore are susceptible to *K. brevis* allelopathy, contrasting their responses with those of nearshore phytoplankton. Since allelopathy is likely to be most effective when members of an allelopathic species congregate at high population density (Jonsson *et al.*, 2009), we surmised that *K. brevis* allelopathy could be targeted toward nearshore competitors. Alternatively, because plankton community members found offshore would likely only encounter dense populations of *K. brevis* when blooms are advected back offshore during export (or bloom termination) (Brand and Compton, 2007; Vargo, 2009), offshore competitors may be more susceptible to allelopathic compounds given that they are less likely to have evolved resistance.

To test these competing hypotheses, we used laboratory-based experiments to compare the susceptibility of eight diatom species (four commonly found nearshore and four more typically offshore in the Gulf of Mexico) to *K. brevis* allelopathy. We also measured responses of natural phytoplankton assemblages sampled from offshore habitats to *K. brevis* allelopathic exudates previously shown to inhibit growth of nearshore diatoms.

METHOD

Organisms

Phytoplankton were obtained from the National Center for Marine Algae (NCMA; formerly the Provasoli-Guillard National Center for Culture of Marine Phytoplankton) and stock cultures were maintained in natural Maine seawater purchased from NCMA (35 ppt) amended with L1 + Si media (Guillard and Hargraves, 1993). For laboratory-based experiments (see below), phytoplankton were grown in L1 + Si made with artificial seawater (35–36 ppt). Diatoms used were *Amphora* sp. CCMP 129, *Asterionellopsis glacialis* strain CCMP 137, *Chaetoceros affinis* strain CCMP 159, *Leptocylindrus danicus* strain CCMP 1856, *Odontella aurita* strain CCMP 1796, *Rhizosolenia* cf. *setigera* strain CCMP 1694, *Skeletonema grethae* strain CCMP 775, *Stephanopyxis turris* strain CCMP 815, and *Thalassiosira* sp. strain CCMP 1055. The dinoflagellate *K. brevis* strain CCMP 2228 and strain TxB3 (obtained from L. Campbell) were grown in the same conditions above, in L1 media without silicates. Strain 2228 was originally isolated from the Mote Marine Laboratory dock in Sarasota, FL, USA (NCMA); whereas strain TxB3 was isolated off South Padre Island, TX, USA (Errera *et al.*, 2010). Cultures were grown at 21°C in a Percival incubator with irradiance of 100–145 μmol m^{-2} s^{-1} (Biospherical Instrument QSL2100) set to a 12 : 12 light/dark cycle. For some experiments, cultures were grown in an environmental chamber at 22°C fitted with Philips Universal/Hi-Vision fluorescent bulbs set to a 12 : 12 light : dark cycle with an irradiance of 75–120 μmol m^{-2} s^{-1}.

Phytoplankton cultures were monitored using *in vivo* fluorescence with a Turner Trilogy fluorometer equipped with an *in vivo* fluorescence module in relative fluorescence mode. Counts of either live cells or cells preserved with Lugol's solution were performed with an inverted Olympus IX50 inverted microscope using a Palmer–Maloney settling chamber or an Utermöhl settling

chamber for less-concentrated samples (see field assemblage experiments below). To enumerate *K. brevis* cells, a FlowCAM autoimager was used with diluted samples preserved with Lugol's solution (Fluid imaging, Inc.; 100-μm flow cell, 0.4 mL min^{-1}, autoimage rate of 16 fps). Growth was calculated using the following equation:

$$\%\,\text{growth} = \frac{\text{cell concentration}_F - \text{cell concentration}_I}{\text{cell concentration}_I} \times 100, \quad (1)$$

where cell concentration_I is the initial cell concentration of phytoplankton, and cell concentration_F is the cell concentration of phytoplankton at the final sampling point. We did not calculate specific growth rate in our experiments because (i) we only generated cell count data for initial and final time points. Thus, we could not confidently determine the growth stages of the phytoplankton during our experiments, and calculating specific growth rate from such data would be inaccurate (Wood *et al.*, 2005). (ii) *In vivo* fluorescence, which was measured at multiple time points, did not reliably predict cell counts due to sublethal effect of *K. brevis* allelopathy on cellular fluorescence.

To normalize the growth of phytoplankton relative to controls in the following experiments, the following equation was used:

$$\text{relative}\,\%\,\text{growth} = \left(1 - \frac{\text{mean}\,\%\,\text{growth of controls} - \%\,\text{growth treatment}}{\text{mean}\,\%\,\text{growth of controls}}\right) \times 100 \quad (2)$$

Extraction of allelopathic compounds from *K. brevis* cultures

Extracellular extracts of cultures of *K. brevis* 2228 and TxB3 were generated for use in experiments with natural plankton assemblages in 2011 (see below). Cultures were extracted in exponential phase after 7–10 days (cell concentrations ranging from 1.9 to 2.7 × 10^4 cell mL^{-1}) of growth using a method optimized from Poulson *et al.* (Poulson *et al.*, 2010). Briefly, organic compounds exuded from *K. brevis* cells (grown as 1.1-L batch cultures) were extracted using a mixture of adsorbent lipophilic resins (Diaion HP20/Amberlite XAD 7 in a 1 : 1 mixture). Cultures were incubated with resins for 12–15 h before gentle sieving to separate cells from resin beads, allowing removal of extracellular compounds from *K. brevis* cultures without cell lysis. Compounds were eluted from the resins with high-performance liquid chromatography grade methanol after sterile artificial seawater (35 ppt) and deionized water rinses. Simultaneously, sterile seawater and L1 media were also extracted to generate negative control extracts. Elution solvent (methanol) was removed by rotary evaporation or with a ThermoSavant SpeedVac concentrator. Dried extracts were stored at −20°C until use.

To test the allelopathic potency of *K. brevis* extracts described above, we used a growth inhibition assay with the nearshore diatom *A. glacialis*, previously shown to be sensitive to *K. brevis* allelopathy (Kubanek *et al.*, 2005; Poulson *et al.*, 2010). *Asterionellopsis glacialis* (3-mL culture in L1 + Si media, in 6-mL culture tubes) was exposed to an amount of extract from 3 mL of *K. brevis* culture in early stationary growth (dissolved in dimethylsulfoxide, DMSO), although natural blooms of *K. brevis* would be expected to produce lower amounts of exudates, due to lower natural abundances compared with culture conditions. Growth of *A. glacialis* was calculated based on *in vivo* fluorescence, using Equation (1). Extracts were deemed allelopathic if they significantly reduced the growth of *A. glacialis* compared with *A. glacialis* treated with a seawater control extract in a one-way ANOVA with Tukey post-test. *Karenia brevis* extracts found to be allelopathic toward *A. glacialis* were used in the natural assemblage experiment in 2011 (see below).

Offshore plankton assemblage experiments

Offshore field assemblage experiment with K. brevis *extracellular extracts*

To determine the impact of *K. brevis* allelopathy on offshore competitors, a natural plankton assemblage was exposed to extracellular extracts of *K. brevis* 2228 and TxB3, similar to the field-based experiment in Poulson *et al.* (2010), which tested for effects of *K. brevis* allelopathy on a phytoplankton assemblage from a very nearshore habitat. The current experiment was performed in 2011 off the Louisiana coast, while aboard the *R/V* Endeavor (Cruise EN496; station 017; 28.8532°N, 88.4897°W; ~65 km offshore; surface salinity 22.6 psu, surface temperature 30.3°C). Nutrient concentrations in surface waters were 0.15 μM nitrate, 0.46 μM phosphate and 13.85 μM silicate. The surface plankton community was sampled using a bucket in early afternoon. The community was gently sieved into 18 polycarbonate bottles through a 300-μm mesh to remove mesograzers and large colonies of *Trichodesmium*. Phytoplankton assemblages (125 mL each) were exposed to *K. brevis* or L1 media control extracts (i.e. extract generated from 125 mL of culture or media dissolved in 210-μL DMSO carrier solvent) ($n = 6$ each). Based on a prediction of 6–8% extraction of media components along with allelopathic compounds (see Prince *et al.*, 2008), nutrient concentrations were estimated to be 53 μM nitrate and 0.17 μM phosphate in experimental bottles at the start of

incubations. Replicate bottles were placed randomly in deck incubators with flow-through surface water to maintain ambient temperature and light regimes.

At times 0 and 42 h, 25 mL of each plankton assemblage was sampled and preserved in Lugol's solution for cell counts. For enumerating phytoplankton, 5 mL of each preserved sample was placed in an Utermöhl settling chamber and settled overnight before light microscopy. For each sample, at least 150 cells were enumerated and identified when possible to the genus level (Thomas, 1997). Growth was calculated for the most prevalent members of the community using Equation (1). To compare the effects of *K. brevis* compounds on competitor growth, a one-way ANOVA with Tukey *post hoc* test was used.

Offshore field assemblage experiment with live K. brevis

To determine the impact of chemical exudates released from live *K. brevis* on the plankton community structure in offshore habitats, an additional experiment was performed on the West Florida Shelf in June of 2012. An offshore plankton assemblage was exposed to *K. brevis* cells while physically separated by dialysis membrane, allowing exchange of waterborne chemicals but without contact between *K. brevis* and members of the plankton assemblage. Unlike in the 2011 experiment, this design also allowed for replenishment of *K. brevis* cues over the course of the experiment, which is important since *K. brevis* allelopathic compounds are labile (Prince *et al.*, 2010).

Plankton assemblages were collected while onboard the *R/V* Endeavor (cruise EN509; station 016; 27° 55.29′N, 87° 01.56′W; ~290 km from shore; surface salinity 34.6 ppt; surface temperature 27.8°C). In surface waters, nitrate and phosphate concentrations were 0.037 and 0.13 μM, respectively, whereas silicate concentrations were 1.06 μM. Plankton were collected with a conical phytoplankton net during mid-morning (10 μm mesh, hand tows at ~1 m depth for ~5 min, $n = 4$ tows). For each replicate, 20 mL of concentrated community mix was added to 200 mL of GF/F-filtered surface seawater (collected from this station) in 250-mL polycarbonate bottles resulting in at least a 10-fold concentration of plankton cells, although population densities of plankton were less than those of the natural abundances observed in the 2011 experiment. *Trichodesmium* colonies and copepods were removed by visual picking, although small mesograzers remained.

For treatments, exponentially growing *K. brevis* 2228 cells were concentrated using a 5-μm regenerated cellulose membrane (Millipore) with an Amicon ultrafiltration unit. Cultures were slowly filtered with constant gentle stirring until ~10% of the initial volume remained. This concentrated culture was then diluted to a cell concentration of $\sim 1.5 \times 10^3$ cell mL^{-1} (similar to natural bloom concentrations) with GF/F-filtered seawater to remove excess nutrients. Previously cleaned, sterilized and cut dialysis tubing (SpectraPor 7, 50 kDa molecular weight cutoff) was knotted at one end and filled with 20 mL of *K. brevis* culture and clipped shut. *Karenia brevis*-filled dialysis tubes were then added to bottles containing plankton assemblages. For controls, 20-mL dilute media (ultrafiltered to 10% volume, then reconstituted to initial volume with filtered seawater) was added to dialysis tubes and placed in bottles containing plankton assemblage ($n = 7$ for both treatment and controls). The nutrient content in each experimental bottle was estimated to be 5.8 μM nitrate and 0.46 μM phosphate. Contents of dialysis tubes for both treatments and controls were replaced after 24 h in order to ensure that *K. brevis* cells were healthy throughout the experiment. Bottles were incubated for 48 h in the same manner as the 2011 experiment and community structure was assessed as above.

Laboratory experiments testing sensitivity of offshore and nearshore phytoplankton to *K. brevis* allelopathy

To decouple the responses of offshore diatom species to *K. brevis* allelopathic compounds from competition among species in the mixed communities, we conducted laboratory experiments in which the sensitivity of diatoms to *K. brevis* was assessed, again with physical separation of species but continual exchange of chemical cues and other dissolved constituents. While many phytoplankton species are found both in offshore and nearshore habitats, we considered species to be representative of our Gulf of Mexico offshore communities, based on (i) isolate source, (ii) personal communication (with C. Heil) and (iii) prevalence in the offshore communities described above versus very nearshore communities as determined from direct observations (Poulson *et al.*, 2010; Myers, Poulson-Ellestad, Sieg, pers. observation). These offshore species included: *L. danicus*, *C. affinis*, *Rhizosolenia setigera* and *S. turris*. Species representative of nearshore communities were *Amphora* sp., *A. glacialis*, *O. aurita*, *S. grethae* and *Thalassiosira* sp. To allow exchange of waterborne exudates while avoiding direct contact between *K. brevis* and competitor cells, *K. brevis* was cultured inside a 50 mL "cage," with a 5-μm nylon mesh on one end, inserted into a 100-mL Pyrex bottle. We used mesh instead of dialysis tubing in the laboratory-based experiments because: (i) we were concerned that the small pore size of the dialysis tubing might slow the exchange of waterborne compounds; (ii) we noticed that the 5 μm mesh accomplished the

same physical separation of cells but with much more rapid equilibration of dissolved materials at a much lower cost. This design most similarly resembled our field-based experiment from 2012 (see above), and allowed for the continual replenishment of *K. brevis* exudates over the course of the experiment.

To construct each cage, the closed, tapered end of a polystyrene centrifuge tube was cutoff and 5-μm nylon mesh was heat-sealed over the now open end. A small hole was drilled in the side (at the 30-mL mark) to accommodate a long needle (600 μm inner diameter) in order to sample cultures without disrupting the cage. The cage was then suspended from the top of a bottle representing one experimental unit. When the media level equilibrated, 3 mL of exponentially growing *K. brevis* (strain 2228) was inoculated inside the cages of treatment replicates. For controls, cages were inoculated with 3 mL of L1 media diluted to 65% full media, to mimic growing *K. brevis* culture. Approximately 20 mL of medium was inside the cage at the start of the experiment, with the total volume of 80 mL in each bottle. The biovolume of *K. brevis* and each competitor tested were within one order of magnitude of each other at the beginning of the experiments (*K. brevis* cell concentrations were between 2.2×10^2 and 5.1×10^3 cell mL^{-1}, while competitors ranged from 3.6×10^3 to 9.7×10^4 cell mL^{-1}). Bottles were placed randomly in an incubator, although treatment and control replicates were always kept in similar light conditions in the incubator setups (i.e. in the same shelf space) to reduce potential for variable irradiance to act as a confounding factor.

To monitor the growth of competitors in response to *K. brevis* exudates exchanged through the cage, 3 mL of competitor species culture was sampled through a needle every other day, over a period of 8–10 days. Every other day, cages were gently lifted to mix cultures and ensure transfer of waterborne compounds through the cage mesh and bottles were rearranged randomly in the incubator. We excluded exotic bacteria by sterilizing the mesh cages in an ultraviolet (UV) hood for ~30 min at the start of the experiment, limiting experimental bacteria to those present in our plankton cultures. We did not actively prevent biofilm formation on the mesh surface, although we observed no clogging of any mesh screens upon visual inspection. *In vivo* fluorescence was measured as a proxy for cell concentration. Since *R. setigera* cells are larger than >300 μm, this species was sampled by removing the cage and sampling through a sterile pipette in order to avoid breaking cells. On the first and last day of the experiment, 3 mL of *K. brevis* culture was sampled and preserved in Lugol's solution for later enumeration as described earlier.

In order to determine if there were any differences in *in vivo* fluorescence between treatments and their respective controls over the course of the experiment, growth curves generated from fluorescence data in the co-culturing assays were analyzed with linear or non-linear regression with an *F*-test (Kubanek *et al.*, 2005). To determine if *in vivo* fluorescence differed between *K. brevis* exposed and media exposed phytoplankton at any specific sampling point, we compared fluorescence values with an unpaired *t*-test. Percent growth was calculated with Equation (1) using cell concentrations determined from visual counts of plankton samples taken at initial and final time points (see above); relative percent (growth normalized to controls) was calculated with Equation (2). Comparison of percent growth and relative percent growth of nearshore and offshore competitors was done using a two-tailed unpaired *t*-test.

RESULTS

Responses of offshore plankton assemblages to chemical cues from *K. brevis*

In two separate years at different offshore locations within the Gulf of Mexico, phytoplankton assemblages dominated by diatoms were not negatively impacted by *K. brevis* exudates (Figs 1 and 2). Extracellular extracts used in the first experiment with offshore assemblages were generated from two *K. brevis* strains originally cultured from Gulf of Mexico blooms, and were confirmed in laboratory experiments to be allelopathic toward at least one nearshore diatom, *A. glacialis* (exudates of *K. brevis* strain 2228 caused 38% growth reduction, exudates of *K. brevis* strain TxB3 caused 75% reduced growth of *A. glacialis*; data not shown). Exposure for 42 h to each of these mixtures of *K. brevis* allelopathic compounds caused slight stimulatory effects on centric diatoms when analyzed as a group ($P = 0.050$; Fig. 1A). Among the centric diatoms growth of members of the most abundant genus, *Chaetoceros,* was unaffected by *K. brevis* exudates relative to media extract controls whereas growth of members of the genus *Leptocylindrus* was stimulated relative to controls ($P = 0.57$ for *Chaetoceros*; $P < 0.0001$ for *Leptocylindrus*; Fig. 1C and D). The enhancement of *Leptocylindrus* spp. growth by exudates of *K. brevis* strain TxB3 was most dramatic: populations increased by 182% relative to media controls ($P = 0.0009$) while exposure to compounds from the other *K. brevis* strain (2228) marginally stimulated the growth of *Leptocylindrus* spp. (106% enhancement of growth versus controls; $P = 0.080$; Fig. 1D). Additional diatom genera within the centric diatom group included *Thalassionema*, *Rhizosolenia*

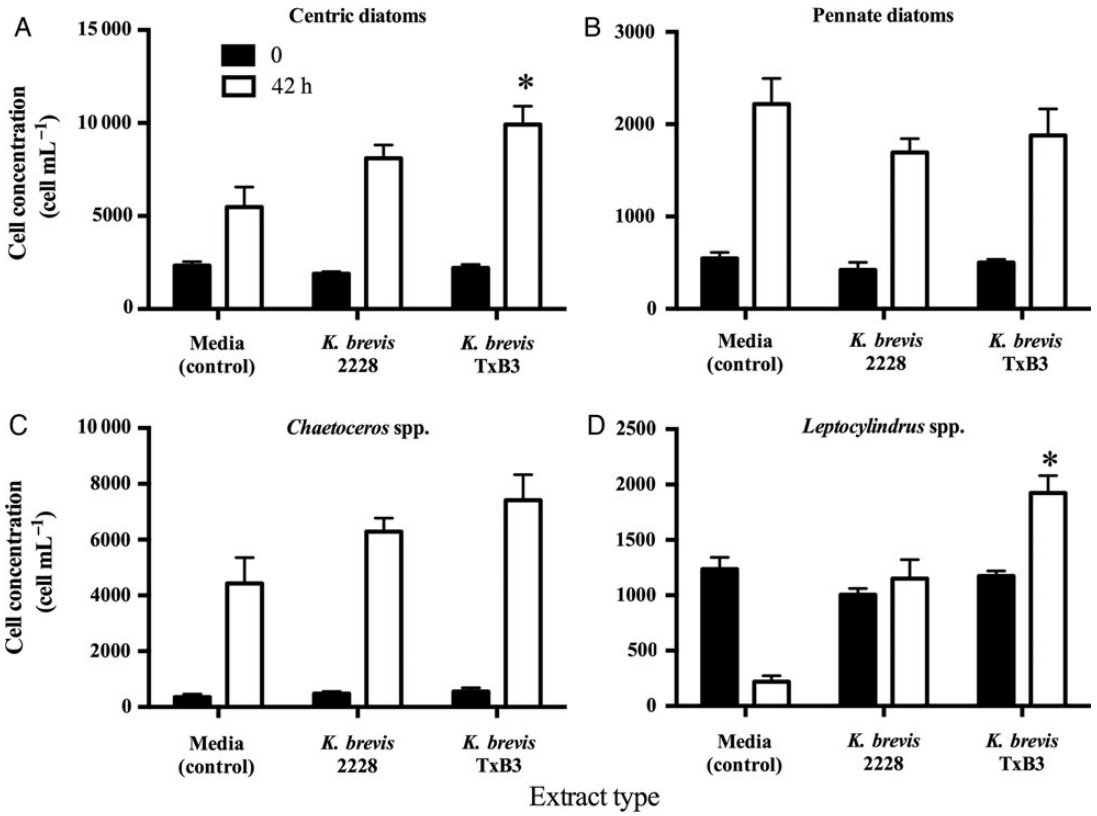

Fig. 1. Effects of *K. brevis* extracellular extracts on Gulf of Mexico field assemblages of (**A**) centric diatoms and (**B**) pennate diatoms, collected offshore in 2011. The growth of dominant community members (**C**) *Chaetoceros* spp. and (**D**) *Leptocylindrus* spp. in response to control extracts of media ("Media"), *K. brevis* strains 2228 ("*K. brevis* 2228") and TxB3 ("*K. brevis* TxB3") is also shown. Asterisks indicate significant differences in growth between treatments and controls after 42 h ($n = 6$; one-way ANOVA with Tukey post-test, $P \leq 0.05$). Error bars represent ± 1 SEM.

and *Guinardia*. Pennate diatoms (dominated by *Pseudo-nitzschia* spp. and *Cylindrotheca* spp.) appeared neither stimulated nor inhibited by *K. brevis* exudates ($P = 0.41$; Fig. 1B), whereas effects on the much-less-abundant offshore dinoflagellates were more variable (dinoflagellates accounted for ~2% of total cells observed; data not shown). In general, this natural offshore phytoplankton assemblage experienced either neutral or weakly stimulatory effects from exposure to *K. brevis* exudates that were previously shown to be allelopathic to at least one nearshore diatom *A. glacialis* (see above).

In a second experiment with offshore phytoplankton assemblages from a different location and time period, diatoms again dominated the community; in particular *Rhizosolenia* spp., *Chaetoceros* spp. and *Pseudo-nitzschia* spp. Additionally, *Leptocylindrus* spp., *Guinardia* spp., *Thalassionema* spp. and *Cylindrotheca* spp. were present. In this experiment, we exposed field-collected assemblages to exudates from live *K. brevis* contained in dialysis tubing, which prevented contact between the two species but allowed exchange of waterborne chemicals throughout 48 h. This design also allowed the ongoing production and release of allelopathic compounds by live *K. brevis*, which was not possible in the first experiment with extracellular extracts. The growth of both centric and pennate diatoms was significantly stimulated by the presence of live *K. brevis* cells ($P = 0.014$; $P = 0.012$, respectively; Fig. 2A and B). When considered separately, however, the growth of dominant centric diatom genera, specifically *Rhizosolenia*, *Chaetoceros* and *Leptocylindrus* was not significantly affected by *K. brevis* exudates although trends toward stimulation were observed ($P = 0.86$, $P = 0.12$ and $P = 0.29$, respectively; Fig. 2C–E). Of the pennate diatoms, *Pseudo-nitzschia* spp. dominated the assemblages, significantly stimulated by 42% by exposure to *K. brevis* relative to controls ($P = 0.0045$). Dinoflagellates, which represented a very small proportion of the phytoplankton community (<1%), were not affected by *K. brevis* exudates (data not shown). Overall, exposure to *K. brevis* exudates caused stimulatory effects on the growth of diatoms, mainly *Pseudo-nitzschia* spp., from this plankton assemblage.

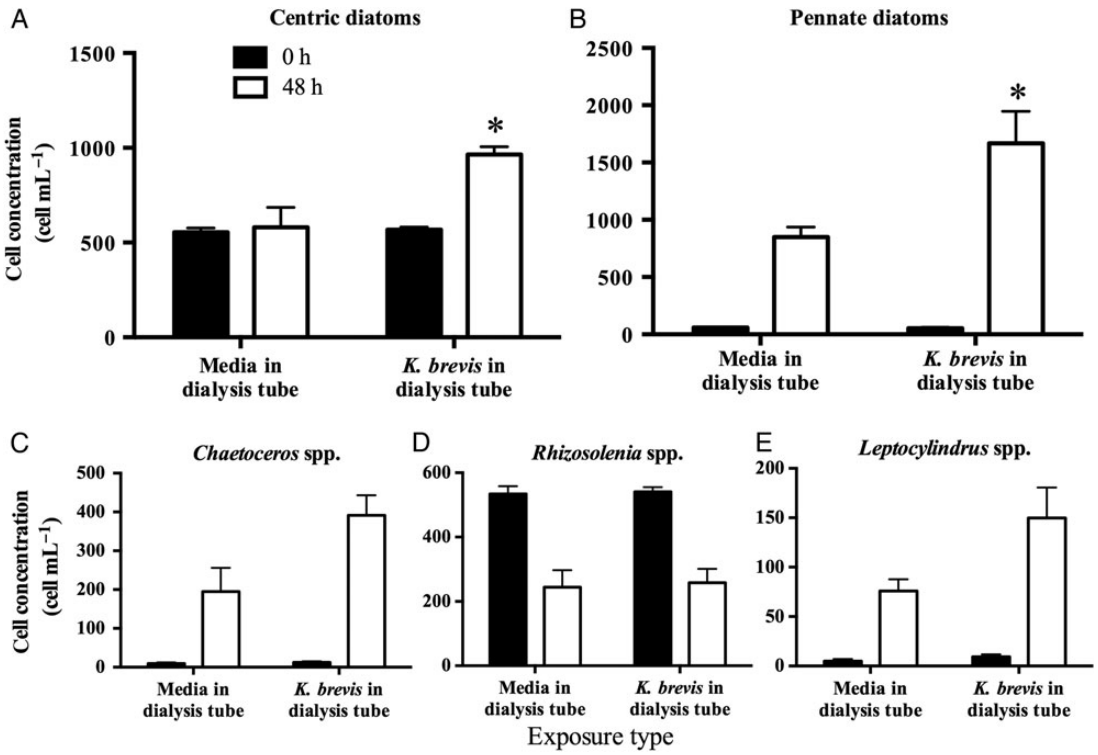

Fig. 2. Effects of exudates from live *Karenia brevis* on Gulf of Mexico field assemblages of (**A**) centric diatoms and (**B**) pennate diatoms, collected offshore in 2012. The growth of dominant community members (**C**) *Chaetoceros* spp., (**D**) *Rhizosolenia* spp. and (**E**) *Leptocylindrus* spp. in response to *K. brevis* exudates exuded through dialysis tubing is also shown. Asterisks indicate significant difference in growth between treatments and controls after 48 h ($n = 7$; unpaired t-test, $P \leq 0.05$). Error bars represent ± 1 SEM.

Responses of offshore and nearshore competitors to *K. brevis* in pair-wise laboratory experiments

Of four cultured diatoms known to occur offshore in the Gulf of Mexico (three of which were also dominant in our offshore field assemblages), none were stimulated by *K. brevis* exudates. Growth (calculated from initial and final cell counts) of *C. affinis, L. danicus* and *S. turris* was not significantly impacted by exposure to *K. brevis* exudates for 8–10 days although all three species displayed a trend toward sensitivity to allelopathy (unpaired t-test; $P = 0.14$, $P = 0.11$ and $P = 0.17$, respectively; Fig. 3). Growth of *Rhizosolenia setigera* was not significantly affected by exposure to *K. brevis* exudates for 10 d ($P = 0.59$; Fig. 3). However, *in vivo* fluorescence of *R. setigera* was significantly enhanced by *K. brevis* after 8 days, relative to controls (unpaired t-test; $P = 0.018$; Fig. 4). Similarly, *in vivo* fluorescence of *S. turris* was enhanced by exposure to *K. brevis* by the end of the 10 d experiment (unpaired t test $P = 0.017$; Fig. 4), despite a substantial, albeit not significant, 43% reduction in *S. turris* growth over 10 days ($P = 0.17$; Fig. 3). This suggests that although extremely variable, per-cell fluorescence for these two competitors was enhanced when exposed to *K. brevis* exudates. Overall, in laboratory-based experiments, offshore competitors were either unaffected or displayed slightly reduced growth, and enhanced *in vivo* fluorescence by exposure to *K. brevis* exudates (Figs 3 and 4), in contrast to our observations using field assemblages (Figs 1 and 2).

Nearshore competitors exhibited either resistance or sensitivity, in a species-specific manner, when exposed to waterborne exudates of *K. brevis* (Figs 3 and 4). Growth of *A. glacialis* was reduced by 73% after exposure to *K. brevis* allelopathy (unpaired t-test; $P = 0.011$; Fig. 3) while *S. grethae* displayed a 47% reduction in growth after exposure to *K. brevis* exudates, although this trend was not significant ($P = 0.24$). Given that *in vivo* fluorescence of *A. glacialis* and *S. grethae* remained nearly identical for treatments and controls for 8–10 days in these experiments (Fig. 4), it appears that these species experienced enhanced per-cell fluorescence, similar to that observed for offshore competitors. The other nearshore species, *Amphora* sp., *O. aurita* and *Thalassiosira* sp., were resistant to *K. brevis* allelopathy, as their growth was not reduced compared with dilute media controls ($P = 0.85$, $P = 0.79$ and $P = 0.39$, respectively; Fig. 3). No significant effects on *in vivo* fluorescence were observed for *O. aurita* and *Thalassiosira* sp., whereas the *in vivo* fluorescence of

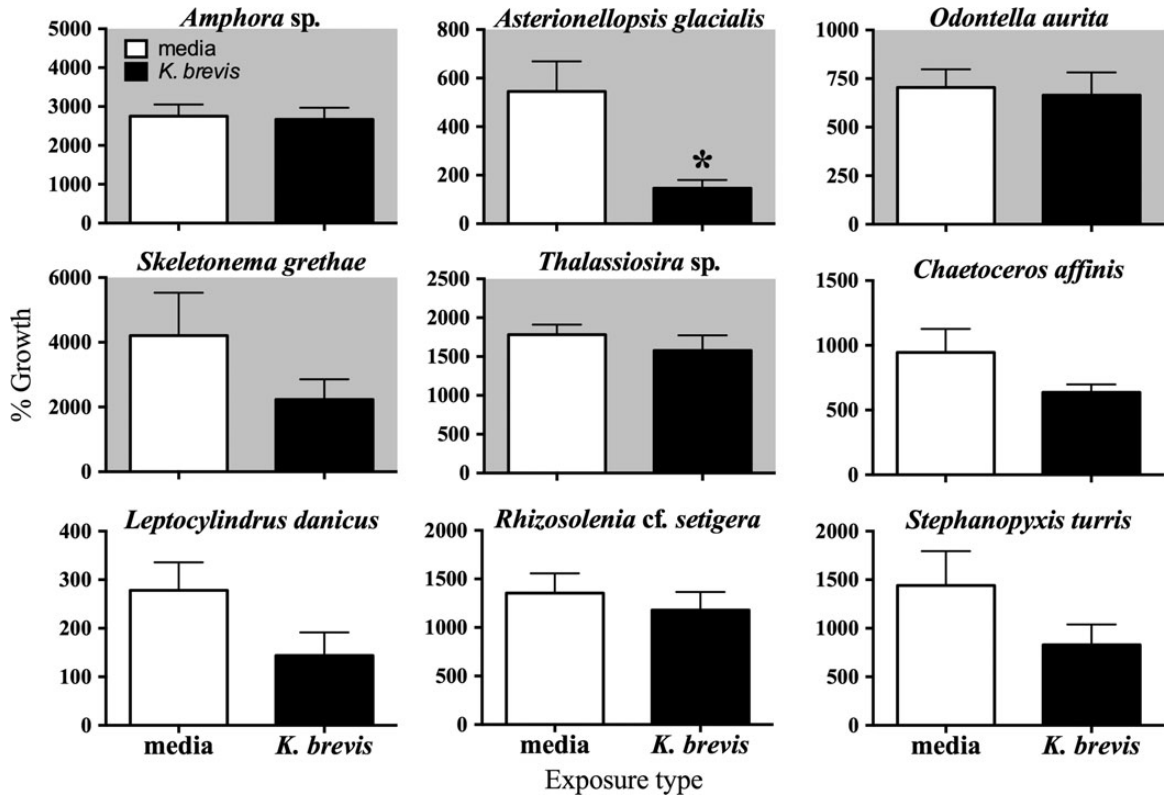

Fig. 3. Growth of competitors exposed to exudates from live *Karenia brevis* (black bar) or dilute media controls (white bar) across 5-μm mesh in pair-wise, laboratory experiments. For each competitor species, growth was calculated from initial and final cell concentrations counted under microscope ($n = 3–6$). Phytoplankton known to occur nearshore in the Gulf of Mexico are denoted by gray background; offshore species by white background. Asterisks indicate significant differences between treatments and controls ($n = 3–6$; unpaired *t*-test). Error bars represent ± 1 SEM.

Amphora sp. exposed to *K. brevis* exudates was significantly enhanced on the final day of the experiment (unpaired *t*-test, $P = 0.021$, Fig. 4).

When considering overall growth for all eight cultured diatom species exposed versus not exposed to *K. brevis* exudates for 8–10 days, the responses of offshore diatoms did not significantly differ from those of nearshore diatoms ($P = 0.72$; Fig. 5), indicating that offshore and nearshore diatoms, as a group, exhibit a similarly variable response to *K. brevis* allelopathy. With ongoing exposure to *K. brevis* exudates for 8–10 days, the overall mean growth of both the nearshore and offshore competitor species was reduced 35 ± 17 and 29 ± 31% relative to controls, respectively (Fig. 5).

DISCUSSION

Our field results suggest that offshore diatoms are either resistant to or stimulated by *K. brevis* exudates when present in complex assemblages (Figs 1 and 2). The stimulatory effects of *K. brevis* are evident for multiple diatom species from two different offshore communities separated by hundreds of kilometers in the Gulf of Mexico and separated temporally across two different years (Figs 1 and 2), suggesting relative robustness in offshore diatoms' responses to *K. brevis* allelopathy. In a previous, similarly designed study, members of a phytoplankton assemblage from a nearshore habitat were also resistant to or stimulated by *K. brevis* allelopathic extracts (Poulson *et al.*, 2010). Additionally, there was no significant impact of *K. brevis* allelopathy on the growth of offshore species in our pair-wise laboratory experiments, although three species tended toward sensitivity to *K. brevis* (Fig. 3). The *in vivo* fluorescence of two offshore species (*R. setigera* and *S. turris*) was slightly enhanced by *K. brevis* during the experiment (Fig. 4), suggesting a subtle, sublethal impact of *K. brevis* exudates on the physiology of these offshore competitors. Because *K. brevis* allelopathic compounds are known to reduce photosynthetic efficiency and disrupt energy metabolism of sensitive competitors (Prince *et al.*, 2008; Poulson-Ellestad *et al.*, 2014), it is plausible that the enhanced per-cell fluorescence of *R. setigera* and *S. turris* resulted from

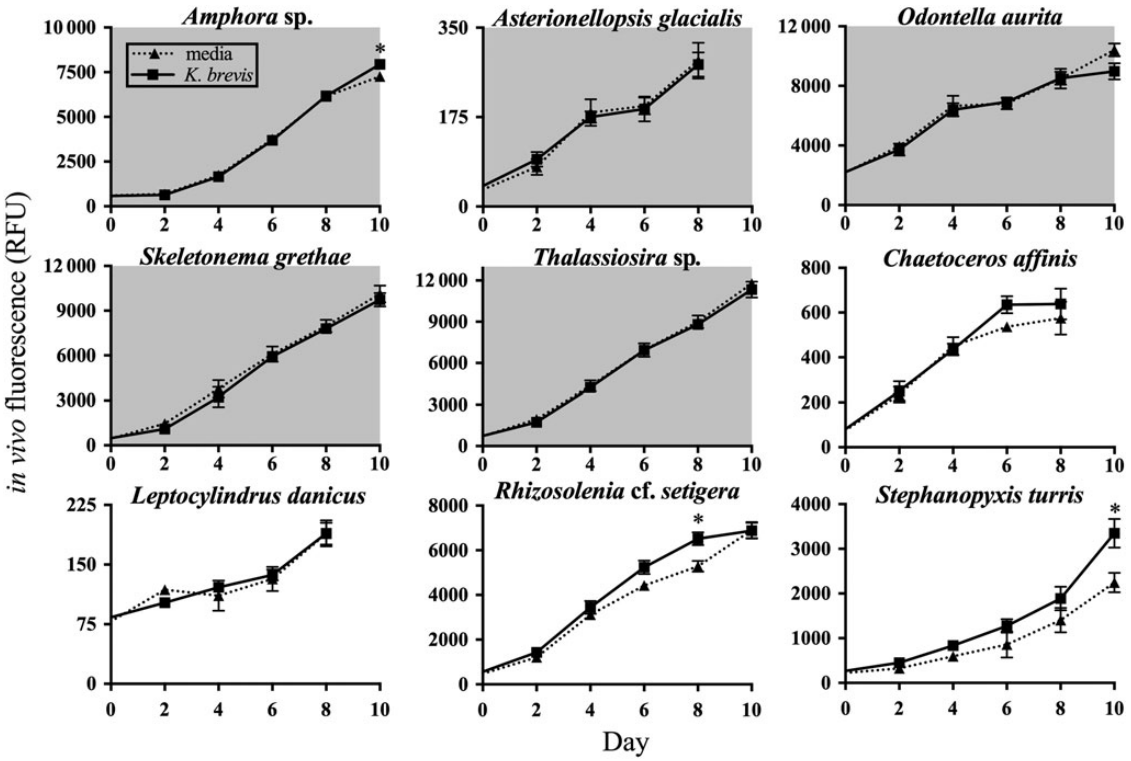

Fig. 4. Competitor *in vivo* fluorescence over 8–10 days exposure to exudates from live *K. brevis* (solid line) versus dilute media (dashed line) in laboratory-based, pair-wise experiments. Phytoplankton known to occur nearshore in the Gulf of Mexico are denoted by gray background; offshore species by white background. Asterisks indicate significant differences between treatments and controls ($n = 3–6$; unpaired *t*-test). Error bars represent ± 1 SEM.

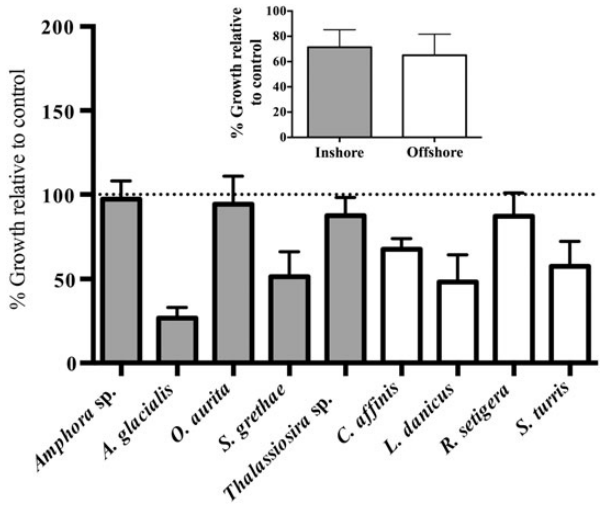

Fig. 5. Effects of exposure to *K. brevis* exudates on nearshore (gray bars) and offshore (white bars) competitor growth after co-culture for 8–10 days with *K. brevis*, separated by 5-μm mesh ($n = 3–6$). Growth equivalent to controls (100%) is indicated by a dotted line. Cells were counted under microscope. Inset: growth of nearshore versus offshore competitor species relative to controls ($n = 5$ nearshore and $n = 4$ offshore species, unpaired *t*-test, $P = 0.72$). Error bars represent ± 1 SEM.

reduced photosynthetic efficiency due to exposure to sublethal doses of *K. brevis* compounds. In addition, growth of *A. glacialis* was significantly reduced by exposure to *K. brevis* exudates while *A. glacialis in vivo* fluorescence stayed the same (Fig. 4), suggesting that *K. brevis* allelopathy lowered the photosynthetic efficiency of individual *A. glacialis* cells. However, because of the multitude of factors which influence *in vivo* fluorescence in growing cultures (e.g. nutrient starvation, light limitation etc.) (Berges and Falkowski, 1998), this possibility remains speculative.

Observations from laboratory experiments in the current study indicate that allelopathic compounds produced by *K. brevis* are potentially antagonistic toward competitors typically found both nearshore and in offshore habitats within the Gulf of Mexico (Fig. 5), but allelopathic effects are likely mitigated by other ecological factors. At least one of the four nearshore diatom species tested, *A. glacialis*, was sensitive to *K. brevis* exudates in the pair-wise co-culture experiment, whereas several other competitors, including those typical of offshore environments, were weakly suppressed or resistant to *K. brevis* (Figs 3–5). However, since the growth of some offshore competitors was stimulated by *K. brevis* exudates in our

field experiments, it is unlikely that allelopathy aids in the formation of *K. brevis* blooms, and is only likely to impact competitors during concentrated bloom events as previously predicted (Jonsson *et al.*, 2009). These subtle effects of *K. brevis* allelopathy are also likely to be overshadowed by a host of other ecological interactions in natural systems, including parasitism, grazing and facilitative interactions, among others (e.g. Poulson *et al.*, 2010).

Overall, competitors appear to respond in species-specific ways to *K. brevis* allelopathy with no obvious pattern when comparing offshore and nearshore phytoplankton (Fig. 5). We found that three offshore competitors displayed marginally (although not significantly) reduced while two species experienced enhanced *in vivo* fluorescence, indicative of physiological stress, when exposed to *K. brevis* (Figs 3–5). Nearshore species ranged from strong sensitivity to *K. brevis* allelopathy (one competitor) to marginal sensitivity (one competitor) to resistance (three competitors). These results led us to reject the hypothesis that species less frequently exposed to dense *K. brevis* blooms are more sensitive to allelopathy, and also to reject an alternative hypothesis that *K. brevis* has evolved targeted allelopathy toward nearshore species. Instead, it appears that the influence of allelopathy is similarly variable in offshore and nearshore communities.

Karenia brevis produces a mixture of unidentified allelopathic compounds (Prince *et al.*, 2010), which may be directly responsible for growth stimulation of offshore competitors in our natural assemblages. Alternatively, the *K. brevis* cues responsible for stimulating the growth of offshore diatoms may differ from those causing inhibitory effects on other competitors. If separate compounds cause stimulatory and inhibitory effects that would indicate that these competitors resist the effects of allelopathic compounds while responding to additional stimulatory compounds. Suikkanen *et al.* (Suikkanen *et al.*, 2005) found that exudates of the cyanobacterium *Nodulara spumigena* stimulated the growth of plankton in natural assemblages, whereas exudates were inhibitory toward competitors in laboratory experiments, suggesting that numerous interactions, as well as the particular nutritional environment, influence the impact of allelopathy in more complex systems. In addition to extra inorganic nutrients delivered into our experiment units, *K. brevis* may stimulate competitor growth directly by providing organic nutrients or compounds readily metabolized by offshore phytoplankton. We also did not assess the bacterial community, which can be greatly impacted by allelopathy among eukaryotic plankton (Uronen *et al.*, 2007; Weissbach *et al.*, 2010) and could play a role in these interactions, specifically in the recycling and transformation of organic nutrients supplied by *K. brevis* or by directly competing with phytoplankton. *Karenia brevis* could also enhance competitors' ability to take up nutrients from the environment by the production of trace metal chelators (Trick *et al.*, 1983; Amin *et al.*, 2009) or alkaline phosphatases (Vargo *et al.*, 2008). The specific mechanisms involved in the observed growth stimulation of offshore competitors in field assemblages are unknown.

In addition to direct stimulation of growth, increases in competitor population density could arise from reduced mortality. Exudates from *K. brevis* could release phytoplankton from grazing or competitive pressures, provide a defense against pathogens or inhibit death-related metabolic pathways (e.g. apoptosis). We only observed small populations of tintinnids and dinoflagellates in our assemblages; however, their presence was not confirmed in all replicates (data not shown). This inconsistent presence or activity of grazers could explain some variability in our field data. The presence of mesograzers in our 2012 field study may have reduced pressure on phytoplankton from micrograzers, obscuring our ability to detect a true effect of allelopathy. In our field assemblage experiments, we did not observe any reductions in growth of the major taxa present (Figs 1 and 2), suggesting offshore diatoms were not stimulated by the death of a competitive-dominant diatom or microplankter although *K. brevis* exudates could have impacted populations of nano- and picoplankton, releasing diatoms from competitive pressures. Alternatively, it is possible that *K. brevis* exudates reduced micrograzer populations or inhibited their feeding behavior (e.g. Hansen, 1995; Tillmann, 2004). High and variable grazing rates have been reported in the northern Gulf of Mexico for micrograzers. For instance Murrell *et al.* (Murrell *et al.*, 2002) reported micrograzer rates from 0.53 to 0.80 day^{-1} during the summer months which could remove approximately half of the plankton standing stock per day. Liu and Dagg (Liu and Dagg, 2003) found that the relative impact of micrograzers (grazing rates of 0.97–1.82 day^{-1}) outweighs that of mesograzers (0.001–0.102 day^{-1}) on phytoplankton communities in Mississippi plume water. High grazing rates of dinoflagellates on diatoms have also been reported (1.7 day^{-1} in the northern Gulf of Mexico) (Strom and Strom, 1996). Thus, any reduction of grazing due to *K. brevis* compounds could have had profound impacts on planktonic community structure and may explain the stimulation of phytoplankton (notably diatoms) in our study. It is clear from these results that the relative impacts of *K. brevis* exuded compounds (i.e. potentially allelopathic compounds, not brevetoxins) on both micro- and mesograzers along with subsequent influences on community composition warrants investigation, as we did not explicitly test this in the current study.

How might it benefit *K. brevis* to stimulate the growth of potential competitors, if *K. brevis* does indeed benefit?

K. brevis could benefit by stimulating growth of certain offshore diatoms, particularly if they harbor nitrogen-fixing symbionts, as do *Rhizosolenia* spp. (Villareal, 1990). *Karenia brevis* could also benefit from fixed nitrogen released from *Trichodesmium* blooms preceding *K. brevis* blooms on the West Florida Shelf, fertilizing *K. brevis* (Lenes *et al.*, 2001; Walsh and Steidinger, 2001). Stimulating these diazotrophs may be a mechanism by which *K. brevis* increases its access to fixed nitrogen. This hypothesis remains to be directly tested, however. Alternatively, there may be no ecological or physiological benefit to *K. brevis* when the growth of other planktonic organisms is stimulated. Our results may simply reflect the lack of selective pressure to produce allelopathic compounds targeted toward offshore diatoms, allowing these competitors to utilize beneficial metabolites produced by *K. brevis*.

K. brevis allelopathy is subtle and inconsistent, displaying intraspecific variability in potency and operating in a species-specific manner. In the current study, diatoms from field-collected offshore assemblages were either stimulated or not impacted by compounds exuded from two Gulf of Mexico strains of *K. brevis* (Figs 1 and 2). In laboratory-based, pair-wise experiments, some offshore competitors (*R. setigera* and *S. turris*) experienced subtle physiological impacts by *K. brevis* exudates although their growth was not significantly reduced (Fig. 3). Previous studies have also demonstrated species specificity of allelopathic outcomes involving *K. brevis* and its competitors (Kubanek *et al.*, 2005). Species-specific effects are a common pattern in studies of allelopathy in the marine plankton (but see Tang and Gobler, 2010). *Alexandrium tamarense* exudates have been shown to impact natural plankton communities in a species-specific manner: some groups of competitors were killed, while others, such as *Leptocylindrus* spp., were only moderately suppressed (Fistarol *et al.*, 2004). In some cases, other organisms were stimulated by these same *A. tamarense* exudates (Fistarol *et al.*, 2004). Weissbach *et al.* (Weissbach *et al.*, 2010) observed variable effects of two *A. tamarense* strains on plankton communities, with negative impacts on competitor abundances observed only with high concentrations of allelopathic exudates. Some species, including *Chaetoceros* spp., were enhanced by addition of nonlytic *A. tamarense* supernatant (Weissbach *et al.*, 2010). Filtrates of *Alexandrium fundyense* strongly suppressed growth of many competitors in natural assemblages, while having a positive effect on the growth of other dinoflagellates (Hattenrath-Lehmann and Gobler, 2011).

Because of the mild effects that *K. brevis* has on competitors, it is likely that any benefits *K. brevis* derives from allelopathy occur primarily during dense blooms, maximizing waterborne concentrations of allelopathic compounds when competition is likely to be especially fierce and when *K. brevis* cells can reap the rewards of reduced competitor growth and compromised physiological state (Lewis, 1986; Jonsson *et al.*, 2009). The subtle effects of *K. brevis* allelopathy are in stark contrast to those of some other allelopathic phytoplankton including *Alexandrium* spp., which are known to cause rapid, dramatic effects on competitor species including cell lysis, in laboratory and field-based studies (Tillmann *et al.*, 2007; Hattenrath-Lehmann and Gobler, 2011). In a mixed assemblage, it may be difficult to decipher the many types of ecological interactions and different forms of competition. Influences of variable nutrient concentrations and other ecological interactions (e.g. grazing, parasitism) likely overshadow the subtle effects of *K. brevis* allelopathy in natural systems (Poulson *et al.*, 2010).

The role of interspecific interactions between planktonic organisms in mediating large-scale ecosystem and biological phenomena is becoming increasingly apparent (Strom, 2008). The current study highlights the potential for chemical cues to structure plankton communities, through mediating species interactions such as competition (e.g. allelopathy), grazing (e.g. defense, predator and prey location, precapture) and even facultative interactions (Roy *et al.*, 2013). However, our study also demonstrates how allelopathy can affect competitors in a complex manner and stresses the need to investigate the role of allelopathy in more natural settings consistent with field conditions, since allelopathy can have unexpected consequences on competitors. Our results suggest that competitors common to nearshore and offshore environments are variably sensitive to allelopathy with several species subtly affected by *K. brevis* exudates. These findings support the hypothesis that allelopathy is not likely to be used by *K. brevis* to support initiation of its blooms, but may play a role in bloom maintenance.

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