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Trichodesmium-derived dissolved organic matter is a source of nitrogen capable of supporting the growth of toxic red tide *Karenia brevis*

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ABSTRACT: Dissolved organic nitrogen (DON) produced by the nitrogen-fixer *Trichodesmium* sp. has the potential to serve as a nitrogen source for the red tide dinoflagellate *Karenia brevis*. Dissolved organic matter (DOM) from laboratory cultures of *Trichodesmium* sp. was isolated, concentrated and then supplied as a nutrient source to *K. brevis* cells collected from the Gulf of Mexico. *K. brevis* abundance increased immediately after *Trichodesmium* sp. cellular exudate (TCE) addition, allowing the population to double within the first 24 h. There was rapid and complete utilization of the TCE DON as well as ~89% of the TCE dissolved organic phosphorus (DOP). Additionally, terminal restriction fragment length polymorphism (TRFLP) was used to assess the bacterial community response to the addition of TCE . The number of bacterial operational taxonomic units (OTUs) initially increased after the TCE DOM addition, but decreased as *K. brevis* reached its maximum abundance. Electrospray ionization mass spectrometry (ESI-MS) and Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) were used to chemically characterize the DOM. Approximately 25% of compounds disappeared within the first 24 h, corresponding to the greatest increase in *K. brevis* abundance. Using FT-ICR MS, 391 DON and 219 DOP potentially bioavailable compounds were characterized. The bioavailable DON compounds were highly reduced and 44% had molar ratios indicative of lipid or protein-like compounds. The changes in DON concentration and compound composition show that *Trichodesmium* sp. provides a sufficient source of nitrogen to directly or indirectly support *K. brevis* blooms.

KEY WORDS: Red tide · *Trichodesmium* · *Karenia brevis* · DON · FT-ICR MS · ESI-MS

INTRODUCTION

Blooms of the toxic red-tide dinoflagellate, *Karenia brevis* (Davis) Hansen and Moestrup (previously known as *Gymnodinium breve* (Davis) and *Ptychodiscus brevis* (Davis) Steidinger), occur frequently on the West Florida Shelf in the subtropical waters of the Gulf of Mexico (GOM). *K. brevis* blooms occur annually and account for a significant proportion of the annual carbon fixation within the GOM (Vargo et al. 1987). Non-bloom concentrations of *K. brevis* are ubiquitous throughout the eastern

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GOM and are also common in the South Atlantic Bight (Geesey & Tester 1993, Tester & Steidinger 1997). Blooms of *K. brevis* (>1000 cells l^{-1}) have been observed in 28 of the 42 years between 1957 and 1998 (Walsh & Steidinger 2001). The frequency and cause of *K. brevis* blooms is important because this species produces a potent neurotoxin, brevetoxin. Brevetoxin has been associated with numerous negative water-quality impacts including marine mammal mortalities, extensive fish kills, human respiratory irritation, and illness in shellfish consumers (reviewed in Kirkpatrick et al. 2004, Landsberg et al. 2009, Fleming et al. 2011, Brand et al. 2012) as well as adverse socioeconomic problems. For example, economic losses associated with *K. brevis* blooms can reach $6.5 million per month to the tourism industry alone (2007 US dollars; Larkin & Adams 2007).

Despite being an important component of the GOM phytoplankton community, the ecology of *Karenia brevis* has been difficult to describe given that blooms generally develop 18 to 74 km offshore (Steidinger 1975, Steidinger & Haddad 1981). In the oligotrophic waters of the GOM, dissolved inorganic nitrogen (DIN) concentrations approach zero and as a result dissolved organic nitrogen (DON) has been hypothesized to play an important role in *K. brevis* nutrition (Baden & Mende 1979, Walsh & Steidinger 2001, Vargo et al. 2008). While correlations have been made between some *K. brevis* blooms and riverine flow, these relationships are not universal and appear locally dependent (Dixon & Steidinger 2004). Although riverine nutrients may supply some of the nitrogen (N) required to support coastal blooms, it is likely only one of many sources of N supporting offshore blooms. Additionally, calculations based on nutrients provided from groundwater, riverine, upwelling and atmospheric inputs together cannot account for the total N required to sustain large blooms that reach concentrations of 1×10^7 cells l^{-1} (Walsh & Steidinger 2001, Mulholland et al. 2004, Walsh et al. 2006). Since allochthonous nutrient sources fall short of the amount of N required for growth, studies have focused on autochthonous nutrient sources.

One potentially important autochthonous source of N is *Trichodesmium* sp. (hereafter referred to as *Trichodesmium*), a diazotroph which has traditionally been considered the most ecologically important N_2 fixing cyanobacteria in the oceans (Carpenter & Romans 1991, Capone et al. 1997). As *Trichodesmium* fixes N_2 gas from the atmosphere, it releases fixed N into the surrounding water in the form of ammonium (Mulholland & Capone 2001) and DON (Glibert & Bronk 1994, Mulholland et al. 2004), where it can then be used as a nutrient source by other organisms. An estimated 50% of this recently fixed N is released as DON (Capone et al. 1994, Glibert & Bronk 1994). In the GOM, background DON concentrations have been observed to increase by a factor of 3 or 4 (15 to 20 µM N) in water where *Trichodesmium* has bloomed (Lenes et al. 2001). The bulk of the DON appears to be released as highly labile amino acids (Capone et al. 1994). Calculations based on *Karenia brevis'* N demand show that DON released by *Trichodesmium* can represent a significant fraction of the N required for growth (Mulholland et al. 2004, Walsh et al. 2006).

It is also common for *Trichodesmium* and *Karenia brevis* to co-occur, and correlations between *Trichodesmium* presence and *K. brevis* bloom events have been observed (Lenes et al. 2001, Walsh & Steidinger 2001, Walsh et al. 2006, Brand et al. 2012). Isotopic assessments of natural *K. brevis* populations reveal relatively low $\delta^{15}N$ signatures indicating the use of 'new' or recently fixed N (Havens et al. 2004). Uptake of ^{15}N-labeled DON from *Trichodesmium* cultures to a natural non-axenic *K. brevis* population demonstrates that N produced by *Trichodesmium* can be used as a nutrient source by *K. brevis* either directly or indirectly through bacterial remineralization (Bronk et al. 2004). Although there are a number of studies investigating the bioavailability of known DON compounds like urea and amino acids, no studies have evaluated what proportion of *Trichodesmium*-derived DON is bioavailable and what compounds within the uncharacterized DON pool are utilized. With as much as 75% of all DON compounds in the surface ocean remaining uncharacterized at the compound level (reviewed in Aluwihare & Meador 2008), defining and quantifying this pool in terms of its liability is essential for accurate N and C cycle estimates.

Bacterial populations within *Karenia brevis* blooms are also important to consider because bacteria serve as a nutrient source and sink, as well as potential predators and prey. In oligotrophic systems the balance between nutrient regenerators and competitors may be crucial to bloom initiation and proliferation (Bronk et al. in press). Bacteria liberate nutrients from dissolved organic matter (DOM) that are otherwise not available to phytoplankton and can provide a direct source of nutrients through phagotrophic grazing (bactivory) by some heterotrophic harmful algal species (e.g. Stoecker et al. 2006). Finally, algicidal bacteria may aid in bloom termination (Doucette et al. 1999).

While we know that bacteria–algal interactions are common in aquatic systems, especially among harmful algal species, there is relatively little known about the role bacteria play in *Karenia brevis* growth (Kodama et al. 2006, Jones et al. 2010, Brand et al. 2012). *K. brevis* has rarely, if ever, been successfully cultured axenically, suggesting that bacteria may be important for *K. brevis* growth. Nutrient limitation likely plays a key role in bacterial–*K. brevis* interactions (Mayali & Doucette 2002). While laboratory experiments provide insight into potential bacterial–algal interactions, they often cannot mimic the microbial dynamics that occur in nature.

The objectives of this study were 4-fold: (1) to investigate how DON produced by *Trichodesmium* affected the growth of *Karenia brevis*; (2) to determine what proportion of DON produced by *Trichodesmium* was bioavailable; (3) to chemically characterize the DOM produced by *Trichodesmium* and consumed by the *K. brevis*-dominated microbial community; and (4) to determine whether bacterial community composition changes with *K. brevis* growth. To achieve these objectives, DOM from *Trichodesmium* cultures was concentrated and supplied as a nutrient source for a natural *K. brevis* bloom. The changes in the responses of *K. brevis* abundance and nutrient concentrations and composition were monitored for one bloom event.

MATERIALS AND METHODS

Trichodesmium culture exudates were collected, concentrated and then supplied as a nutrient source for a field population of *Karenia brevis* in a 9 d bioassay experiment. The experiment consisted of 4 bioassay treatments run in triplicate. The treatments were: control with no N addition, +nitrate, +urea, and +*Trichodesmium* cellular exudates (+TCE).

Trichodesmium culture concentrates

Trichodesmium IMS101 cultures were grown on low nutrient YBCII media (Chen et al. 1996) in 10 l acid-washed (12% H_2SO_4 with repeated deionized water [DIW] rinses) polycarbonate carboys. Cultures were maintained at 26°C on a 12:12 h light:dark cycle (85–100 µmol quanta m^{-2} s^{-1}) with constant aeration. *Trichodesmium* cultures were harvested by filtering the culture onto combusted (500°C for 4 h) Whatman GF/F filters (nominal pore size of 0.7µm) to separate *Trichodesmium* cells from the exudates. The TCE was sub-sampled for nutrient analyses (dissolved organic carbon [DOC], total dissolved N [TDN], nitrate + nitrite, ammonium, and phosphate) and immediately frozen in 1 l aliquots for salt removal and DOM concentration.

TCE was concentrated and de-salted using a tandem ultra filtration (UF) and solid phase extraction (SPE) method (Simjouw et al. 2005). Briefly, a 5.3 l sample of TCE filtrate was extracted using a stirred ultra filtration cell (Millipore model 8200) fitted with a 1000 Da Millipore regenerated cellulose ultra filtration membrane, in 100 ml increments. Stirred cell extractions were run under N_2 gas to reduce aerobic microbial degradation.

SPE disks were conditioned with two 10 ml aliquots of methanol (MeOH) and two 10 ml aliquots of DIW. Filtrate from the stirred cell extraction was then acidified with 1.7 ml 6N HCl per 1 l sample and filtered onto a 47 mm 3M Empore C18 SPE disk to extract additional compounds. DOM retained on the C18 SPE disks was eluted with two 10 ml aliquots of MeOH. The eluent was then heated at 55°C until all MeOH had evaporated. The DOM retained after the MeOH evaporation step of the SPE extraction was then reconstituted with DIW. Extracted samples in MeOH and post-MeOH evaporation samples reconstituted in DIW were analyzed using electrospray ionization mass spectrometry (ESI-MS) and compared. No significant differences between pre- and post-MeOH evaporation samples were observed, indicating that the level of heat used during the evaporation step did not significantly alter the DOM pool. The 2 DOM fractions (UF and SPE) were then re-combined at their original (pre-concentration) proportions.

With this method, DOM was retained while salts and inorganic nutrients passed through as filtrate. This method was also later used to extract DOM from the bloom water and TCE bioassay samples for mass spectrometric assessments. The salt removal/DOM concentration method does not retain all of the DOM from the initial sample. After the salt removal process, 53% of the DON and 51% of the DOC present in the initial TCE were recovered. The final nutrient concentrations added to the bioassay with the TCE addition were 138 ± 2 µM DOC, 0.1 ± 0.1µM NH_4, 18.9 ± 1.1 µM DON, 0.2 ± 0.1µM PO_4 (plus 2.5 µM PO_4 added to all treatments), and 1.0 ± 0.1 µM DOP. The nitrate + nitrite and urea concentrations within the final TCE addition were below our detection limit (<0.1µM or 0.05 µM, respectively). Therefore, DON accounted for 99% of the total N addition within the +TCE treatments. The salt removal and DOM concentration method was also performed on the TCE

bioassay samples taken at 0, 12, 24, and 72 h. The total recovery of initial DON ranged between 66% and 81% in all TCE time-series treatments. The DOC recoveries were similar to the DON recoveries and ranged between 53 and 77%.

Field sample collection and bioassays

A bloom of *Karenia brevis* was identified via satellite (NOAA 2007) and ship-board observations near Sanibel Island, Florida in the southeastern portion of the GOM at 26° 15.916′N, 82° 01.04′W. Bloom water was collected on 17 October 2007 with a bucket at the surface and was gently filtered through a 64 µm mesh to remove *Trichodesmium* spp. colonies and large zooplankton. Initial cell counts at this site were 10 to 14 million *K. brevis* cells l^{-1}.

Bloom water was then evenly divided into 12 acid-washed (12% H_2SO_4 followed by DIW rinse) 2.5 l polycarbonate bottles. The control treatment contained no additional N. Both nitrate and urea were added to a final 10 µM N addition. Urea and nitrate are both known to be bioavailable to *Karenia brevis* and were used as comparative compounds to determine N limitation and the N preference of this field population. The TCE treatment received a 20 µM N addition of the TCE; the concentration was twice that of the nitrate and urea additions because it was assumed that not all of the DON produced by *Trichodesmium* would be bioavailable. Phosphate was added (2.5 µM) to all treatments to ensure that the bioassays did not become phosphorus (P) limited. Additions were based on documented *K. brevis* growth rates (e.g. Loret et al. 2002) and N uptake rates (e.g. Bronk et al. 2004) available at the time the experiment was designed.

The bioassays were incubated on the ship at ambient temperature and light in a flow-through system shaded by a neutral density screen to reduce light saturation. Incubations began at sunset. Bioassays were sub-sampled for phytoplankton cell counts, bacterial community composition, nutrient concentrations (DOC, TDN, nitrate + nitrite, ammonium, total dissolved phosphorus [TDP] and phosphate), and chemical characterization at time intervals (t) of 0, 12, 24, 72, 144 and 216 h. Nutrient samples were filtered through combusted (450°C for 4 h) Whatman GF/F filters (nominal pore size 0.7 µm) and frozen until analyzed. Duplicate phytoplankton cell count samples were taken per bioassay bottle; one was preserved in Lugol's iodine solution and the other in 2% glutaraldehyde. Cell counts, as well as changes in N concentrations (DIN and DON), were used to assess the bioavailability of N sources to *Karenia brevis* field populations.

Analyses

Karenia brevis cells were identified based on cell morphology and enumerated using an inverted microscope on a bright field setting. DOC and TDN concentrations were measured via high temperature combustion using a Shimadzu 5000A TOC analyzer operating in tandem with an Antek 7000B Total Nitrogen Analyzer (Sharp et al. 1993, 2004). Ammonium, nitrate + nitrite and phosphate concentrations were analyzed using a Lachat 8500 Nutrient Analyzer (Lachat Instruments). TDP was measured via sector field ICP-MS (Field et al. 2007). Both DON and DOP were determined as the difference between the inorganic and TDN or TDP concentration, respectively. Standard deviations (SD) were determined through the propagation of error associated with each subsequent analysis. All nutrient concentrations are presented as the average concentration of triplicate samples ±SD.

Chemical characterization of DOM

DOM from the TCE concentrate, the control at t_0 and the TCE bioassay (at t_0, t_{12}, t_{24}, and t_{72}) were chemically characterized using ESI-MS with single quadrupole detection (Agilent 1100 Liquid Chromatograph/Mass Spectrometer with ESI source). Samples were run under the conditions described in Seitzinger et al. (2005) except for the mobile phase, which was a 50:50 v:v methanol:DIW, and the mass range scanned was 50 to 1000 m/z (specific mass to charge ratio), in the positive ionization mode. Six replicate injections were analyzed per sample to obtain statistically significant replication for interpretation of m/z ion abundance changes. DIW was used for blank correction.

ESI-MS is a soft ionization method, which allows non-fragmented (whole) compounds to be detected. Each unit mass (m/z) represents one or more compounds with the same molecular weight or m/z (Kebarle and Ho 1997). In the positive ionization mode singly charged compounds (m/z ratios) represent the molecular weight of the compound +1 $(MW+H)^+$ (McEwen & Larsen 1997). Compounds detected in the positive mode contain basic functional groups including N heterocycles, alcohols, amines, and amides

(Van Berkel 1997, Seitzinger et al. 2005). ESI-MS allows for the assessment of masses present and tracks changes in each of those masses via changes in the ion abundance (reviewed in Sipler & Seitzinger 2008).

Raw mass spectra data were recorded on Chemstation (version A.7.01) and then statistically analyzed using a custom program designed in C++ where the average ion abundance (±SD) was determined for each mass within the replicate injections. A *t*-test with 95% confidence level was performed on all *m/z* ratios allowing for the retention of all masses with ion abundances significantly different from zero. Instrumental and analytical noise was addressed through blank correction. Ion abundances of *m/z* ratios found within the DIW blank were subtracted from that same *m/z* detected in each sample.

TCE concentrate, bloom water (control) and the TCE bioassay samples were chemically characterized and compared statistically using a standard paired *t*-test with 95% confidence based on ion abundance between 2 samples (i.e. TCE concentrate and control) for each individual *m/z*. The TCE concentrate was diluted with DIW to the same final vol:vol ratio as the TCE bioassay for direct comparison, prior to ESI-MS analysis. To assess temporal changes in the average ion abundance (±95% of the SD) of individual masses during the bioassay experiment, the slope of the line connecting each time point in series was determined. By applying a positive, negative, or zero slope to each line throughout the time series a total time series trend could be applied to each individual mass.

To further investigate the bioavailable DOM, a non quantitative but higher resolution method (Fourier transform ion cyclotron resonance mass spectrometer [FT-ICR MS] with ESI inlet system) was used to detect individual compounds for which molecular formulas were assigned. This higher-resolution technique allows one to identify individual DON compounds and through their detection, in terms of presence and absence, determine which compounds were removed during *Karenia brevis* growth.

Ultra-high resolution samples were analyzed using a hybrid 7 Tesla linear ion trap (LTQ) FT-ICR MS (LTQ FT Ultra, Thermo Electron, Woods Hole Oceanographic Institute Mass Spectrometer Facility) in the positive ionization mode. A larger number of N-containing compounds are found in the positive ionization mode than the negative ionization mode (Van Berkel 1997, Seitzinger et al. 2005), therefore, the present study focuses on those positive mode compounds. Adduct formation in the positive mode can occur, resulting in reduced detection; however, there was no apparent sodium adduct formation within the samples analyzed. Samples were injected into the ESI source at a flow rate of 4 µl min^{-1}. The ESI spray voltage was set at 4.0 kV and the capillary temperature was 265°C. The mass range analyzed was 50 to 1200 *m/z*). Samples were diluted 1:20 sample:MeOH (v:v) and 0.1% formic acid was added to aid with positive mode ionization. Diluted sample solutions were made just prior to analysis. Raw data were collected using Xcalibur v2.0 (ThermoFisher Scientific). Samples were internally calibrated based on methods described in Kido Soule et al. (2010) and the data were processed using a custom Matlab program described in Bhatia et al. (2010). Molecular formula assignments included ^{12}C, ^{13}C, ^{1}H, ^{16}O, ^{32}S, ^{14}N, ^{31}P, and ^{23}Na, elements commonly observed in seawater.

Bacterial community composition

Changes in bacterial community composition were assessed by monitoring 16S rRNA genes coupled to terminal restriction fragment length polymorphism (TRFLP) analysis. DNA was extracted using a modified phenol chloroform method (Sakano & Kerkhof 1998) and purified by cesium chloride centerfugation. *Halobacterium salinarium* DNA (300 ng) was added to 100 ng of sample DNA to aid in visualization of DNA in the cesium gradients. The gradients were then spun for 24 h at 250 000 × *g* in a Beckman Optima TL Ultracentrifuge. The DNA bands were drop-dialyzed on Millipore filters (VSWP 02500) against 10 mM Tris 8.0 for 45 min. Dialyzed DNA was then stored at −80°C until further analysis.

Cleaned genomic DNA was used to amplify the 16S rRNA genes via polymerase chain reaction (PCR). Briefly, 50 µl PCR reactions were set up with 10 ng template and 20 pmol of the universal primer 27 Forward (5′ AGA GTT TGA TCC TGG CTC AG 3′) and the bacterial specific primer 1100 Reverse (5′ GGG TTG CGC TCG TTG 3′) per reaction. The amplification parameters were as follows: 94°C for 5 min followed by 25 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1.1 min and a final extension period of 72°C for 10 min.

TRFLP profiles were generated by restriction enzyme digestion of 15 ng of fluorescently labeled PCR product (6-FAM; Applied Biosystems) with Mnl I (New England Biolab) on an ABI 310 genetic analyzer (Applied Biosystems). Peak detection was set at 25 relative fluorescent units (RFU) and sorted and normalized to a percentage of the sum of the peak areas in the sample. All peaks less than 0.5%

of the total area were removed from analysis to account for difference in sample loading and potential electronic noise. For a more detailed description of these methods please see McGuinness et al. (2006).

The phylogenetic identification of select TRFLP peaks was accomplished through the creation of a clonal library of the 16S SSU genes. Clone libraries of 16S SSU genes were created from unlabeled PCR product using the TA cloning kit as per the manufacturer's instruction. Recombinant clones were screened by TRFLP to associate nearly full-length clones with specific peaks (Babcock et al. 2007). Sequencing was performed using dye terminator kits (ABI) and phylogenetic tree reconstruction utilized the Geneious program and maximum likelihood methods.

The Sorensen similarity index and Simpson's index of diversity were used to compare the bacterial community composition of the TCE time series samples. The Sorensen similarity index was used to assess the relationship between 2 samples based on species present. It is calculated as twice the number of species shared between 2 samples divided by the sum of the total number of species detected in each sample. The Simpson's index of diversity differs from the Sorensen similarity index in that it takes into account not only the number of species but the relative abundance of each species. Both tests have the same interpretational range of 0 to 1, with 1 indicating the greatest degree of similarity or diversity, respectively (Magurran 2004).

RESULTS

Site characteristics

Trichodesmium was observed, but not quantified, in dense patches at the surface and dispersed throughout the water column in the areas within 1 km of the *Karenia brevis* bloom site. The bloom water was dominated by *K. brevis*, which had reached concentrations of 14×10^6 cells l^{-1} at the time of collection, accounting for 99% of the total phytoplankton population. *Karenia mikimotoi* was also present at much lower concentrations (2×10^5 cells l^{-1}). *In situ* DOC, ammonium, nitrate + nitrite, DON, phosphate and DOP concentrations within the bloom were 375 ± 18 µM C, 2.0 ± 0.1 µM N NH_4^+, 0.1 ± 0.1 µM N NO_3^- + NO_2^-, 13.6 ± 1.4 µM N, 0.6 ± 0.2 µM P PO_4^{3-} and 1.1 ± 0.4 µM P, respectively.

Biological and chemical response to nutrient additions

Initial *Karenia brevis* cell counts in all bioassay treatments were $10.2 \pm 0.8 \times 10^6$ cells l^{-1}. The initial bioassay cell counts were lower than bloom concentrations due to dilution associated with the nutrient additions. There was a continuous decline in cell number within the control treatment, but an initial increase in *K. brevis* cell number in all treatments where N was added (Fig. 1). The +nitrate bioassay increased by $4.8 \pm 0.4 \times 10^6$ cells l^{-1} within the first 24 h, taking twice as much time to reach the same maximum concentration as the +urea treatment which had the same amount of added N (10 µM nitrate or urea, respectively). *K. brevis* cell number in the +urea treatment increased by $4.9 \pm 1.1 \times 10^6$ cells l^{-1} within the first 12 h. After the first 12 h, cell number decreased and continued to do so throughout the remainder of the experiment. The largest increase in *K. brevis* cell number occurred when TCE was added, which resulted in an increase of $10.1 \pm 1.4 \times 10^6$ cells l^{-1}, doubling the overall cell concentration within the first 24 h. The TCE treatment achieved twice the overall growth on twice the amount of N (~20 µM DON). The morphology of the remaining populations in all N-containing treatments shifted from the initial large vegetative cells observed on Days 1 to 3, to smaller vegetative, and eventually to reproductive

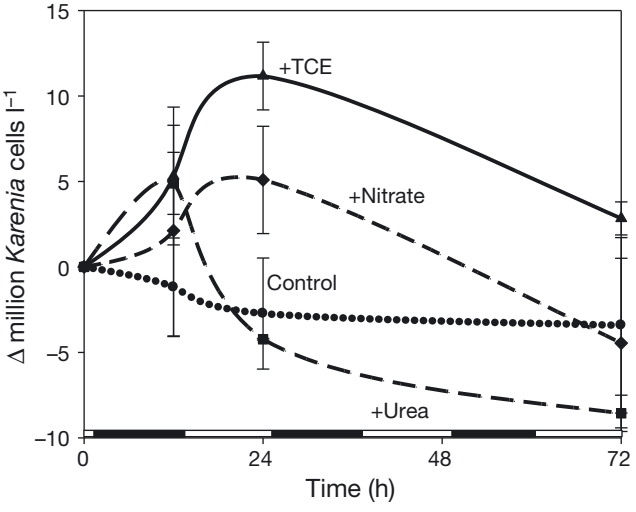

Fig. 1. *Karenia brevis.* Change in cell abundance in the control, +nitrate, +urea, and +TCE bioassays. Points are the average difference in concentration ±SD of triplicate incubations (positive = increased in cell number, negative = decreased in cell number). Initial *K. brevis* cell abundance was $10.2 \pm 0.8 \times 10^6$ cells l^{-1} in all treatments. Black bars along the *x*-axis = periods of darkness. Data from time points after 72 h not shown

cells by Days 6 to 9 as cell numbers decreased (data not shown). These shifts in cell size are likely due to N limitation (Ransom Hardison et al. 2012). Henceforth, we focus on data collected within the first 72 h, before the shift in morphology.

The changes in nutrient concentrations were larger than expected as all N was drawn down from each treatment. An initial decrease in N (DON or nitrate) was observed in all treatments in which N was added (+nitrate, +urea, and +TCE; Fig. 2). DIN, as nitrate only, was only detected in the +nitrate treatment at t_0. The nitrate was quickly consumed to concentrations below our detection limit (<0.1µM) within 12 h of the addition. DON increased over time in both the control and +nitrate bioassays but decreased to background (control) levels (13.1 ± 0.7 µM DON) by 12 h

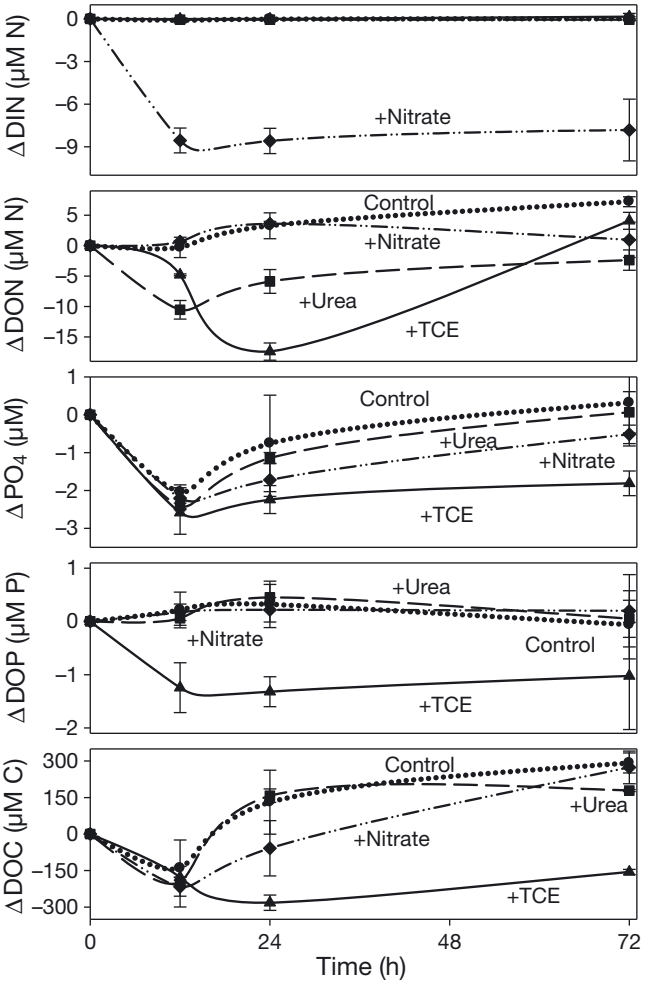

Fig. 2. Changes in the nutrient concentrations in the control, +nitrate, +urea, and +TCE bioassays. Points are the average difference in concentration ±SD of triplicate incubations (positive = nutrient increased in concentration, negative = nutrient decreased in concentration)

in both the +urea and +TCE bioassays, indicating complete utilization of the DON supplied. Considering the recovery of DON during the salt removal process and the use of DON within the +TCE bioassay, at least 53% of DON produced by *Trichodesmium* was directly or indirectly available to the *Karenia brevis* bloom community. There was no significant ($p < 0.05$) decrease in bulk DON concentrations observed in the control, indicating that background DON was likely dominated by compounds that were not readily bioavailable to this community on the time scale investigated.

All of the phosphate supplied (2.5 µM) was consumed in each treatment. Residual phosphate concentrations did not fall below 0.4 ± 0.1 µM (Fig. 2). DOP was only added with the +TCE treatment as a component of the DOM. DOP concentrations in the +TCE treatment decreased by 1.3 ± 0.1 µM between 0 and 24 h, indicating that as much as 89% of the DOP supplied with the TCE DOM addition was consumed by the *Karenia brevis* community. DOC concentrations decreased initially and then subsequently increased after the first 12 h in all treatments except in the +TCE treatment, which continued to decrease in concentration through the first 24 h. This mirrored the increase in cell number and mimicked the observed decreases in DON, DOP and phosphate.

Chemical characterization of DOM

The *Karenia brevis* control (bloom water) and +TCE bioassay samples were compared at the unit mass and compound level (Table 1). The average number of compounds per nominal mass was higher in +TCE bioassay than in the control (13.7 and 10.7 compounds per nominal mass, respectively). Due to the unit mass resolution of ESI-MS, far fewer peaks/masses were observed than under the higher-resolution FT-ICR MS assessment; however, the same general trends and relative proportions of ions between samples were observed. There were 156 masses unique to the TCE concentrate that were not found in the control. However, 173 masses present in +TCE were shared with the control (bloom water), indicating that either (1) microbial byproducts are similar, independent of their species origin, like common amino acids, or that (2) *Trichodesmium* byproducts were present in the *K. brevis* bloom water.

ESI mass spectra collected for t_0, t_{12}, t_{24} and t_{72} of the +TCE bioassays were compared. The semi quantitative capabilities of ESI-MS revealed temporal

Table 1. To compare samples based on the number of compounds detected, *Karenia brevis* bloom water (control), the +*Trichodesmium* cellular exudates (+TCE) bioassay at 0 and 24 h (t_0 and t_{24}) were evaluated based on the number of masses detected using electrospray ionization mass spectrometry (ESI-MS), the number of individual compounds detected using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS), the number and proportion of molecular formulas assigned, and the number of N-containing and P-containing compounds detected. These parameters were also used to evaluate the compounds produced or consumed at +TCE t_{24} and those compounds present in both control and +TCE that were consumed

	Masses detected ESI-MS	Compounds detected FT-ICR MS	Molecular formulas assigned (proportion %)	N-cont. compounds	P-cont. compounds
Control	298	3203	1512 (47)	1093	654
+TCE t_0	329	4498	2115 (47)	1579	887
+TCE t_{24}	395	4805	2223 (46)	1690	935
Produced	189	1326	623 (46)	502	267
Consumed	123	1019	515 (50)	391	219
Present in control and +TCE	31	465	239 (51)	173	107

trends in individual masses. Fifteen percent of all masses detected significantly ($p < 0.05$) increased in ion abundance mirroring the increase in *Karenia brevis* cell number, which suggests that they were biologically produced. In contrast, other masses show no significant change in ion abundance and appear to be refractory, on the time scale of days, to this community. The majority of the masses detected, however, show a variable response, as they significantly ($p < 0.05$) increased and then significantly decreased in ion abundance. Twenty-seven percent of masses significantly decreased in ion abundance over time with 98% of those decreasing masses falling below our detection limits within the first 24 h. Over half of those masses that disappeared within the first 24 h were unique to the TCE concentrate. The DOM fraction that disappeared represents the most interesting fraction, as these corresponded to the observed increase in *K. brevis* abundance; thus we hypothesize that they contributed directly or indirectly to *K. brevis* growth.

The most significant changes in cellular abundance, nutrient concentrations, DOM and individual masses occurred within the first 24 h of the incubation. As a result, we focused on this time frame for higher resolution mass spectrometric assessments and molecular formula assignments. The goal of this effort was to assign molecular formulas to the DON and DOP compounds that likely contributed to *Karenia brevis* growth. Of the compounds detected in any given sample, molecular formulas could be assigned to approximately half of them (Table 1). Similar to the ESI-MS assessment, 23% of all compounds detected at t_0 using FT-ICR MS disappeared in 24 h. While there may be a number of other bioavailable compounds present in this sample, we focus only on those compounds that decreased to below detection as potentially bioavailable compounds.

Twenty-five percent of the DON and DOP compounds present in the initial TCE bioassay sample disappeared within 24 h. Of these potentially bioavailable compounds, 84 contained both N and P. While there was a diverse suite of DON and DOP compounds available, the more reduced compounds (O:C ratio <0.25) made up the greatest percentage (60%) of the consumed DON fraction. This supports earlier work showing that *Karenia brevis* has a preference for reduced N forms (Killberg-Thoreson et al. in press). Forty-four percent of those potentially bioavailable DON compounds fell within the general H:C and O:C molar ratios of biologically produced lipids and proteins. While this is a significant portion of the bioavailable compounds, 45% did not show molar ratio characteristics within these traditional classifications. This is consistent with the fact that not all DOM compounds have been classified (Benner 2002).

Bacterial community composition

The total number of operational taxonomic units (OTUs; one or more bacterial species) was initially stimulated (26 at t_0 to 30 by t_{12}) by the TCE DOM addition but quickly decreased after the first 12 h as nutrient levels decreased and *Karenia brevis* abundance increased. While the number of species increased, the Simpson's diversity index decreased from 0.23 to 0.1. This indicates that specific species may have been stimulated by the TCE additions while other species were not. By t_{24} the total number of observed OTUs detected had decreased to 16; however, the proportion of each OTU (species) had once again become dispersed as indicated by a diversity index of 0.24.

Of the OTUs detected in the TCE bioassays, only 6 represented >10% of the bacterial community at any given time (Table 2). These 6 OTUs represented 20 to

38% of the bacterial community based on the number of OTUs present, and 57 to 81% dominance of the community based on their relative abundance. Only 5 OTUs (OTU 102, 104, 84, 248, and 179) persisted throughout all 3 time points (t_0, t_{12}, and t_{24}). Combined, these 5 persistent OTUs represented between 35% and 65% of the total population (Table 2). Thirteen OTUs were present at both t_0 and t_{12} but disappeared by t_{24}. This decrease in OTUs between t_{12} and t_{24} preceded the observed decrease in *Karenia brevis* abundance.

DISCUSSION

Trichodesmium exudates as a source of N for *Karenia brevis*

A significant amount of N is required for a red tide to reach concentrations of several million cells l^{-1}. DON is the largest pool of fixed N in many aquatic systems (Bronk 2002) and, due to extremely low DIN concentrations in the GOM, DON likely contributes to *Karenia brevis* growth (Walsh & Steidinger 2001). Previous estimates indicate that riverine transport may account for 5 to 20% of the N required to sustain blooms of 3×10^5 cells l^{-1} in certain coastal regions (Vargo et al. 2004). However, bloom concentrations may reach up to 50-fold greater cell density and N demand. By fixing N_2 and subsequently releasing it as ammonium and DON, *Trichodesmium* represents an additional source of N to the ecosystem. Through *Trichodesmium*, *K. brevis* may not only benefit from an initial pulse of N during bloom initiation when cell numbers are low, but more importantly could receive a somewhat continuous source of N throughout their growth in water where the 2 organisms co-occur.

Table 2. Percent dominance of bacterial species representing >10% of the total bacterial community detected in the bioassays where *Trichodesmium* culture exudates were added. Data presented include the operational taxonomic unit (OTU), species (when identified) and the percent dominance of that species or OTU at t_0, t_{12} and t_{24}. Others represent the sum of all species that individually represent less than 10% of the total community. ND: not determined

OTU	Group designation	Percent dominance		
		t_0	t_{12}	t_{24}
102	*Cyanobacteria* (*Synechococcus*-like)	43	18	3
250	*Alphaproteobacteria*	0	10	36
104	*Alphaproteobacteria*	1	1	30
84	ND	16	11	1
248	*Alphaproteobacteria*	4	5	10
113	ND	0	12	1
Others		36	43	19

Within this study we showed that *Karenia brevis* abundance increased when DON and DOP produced by *Trichodesmium* was supplied as a nutrient source to a natural bloom community. The decreases in DON and DOP concentration supplied with the TCE addition within the first 24 h closely mirrored trends observed in *K. brevis* cell abundance. Further, evidence suggests that *K. brevis* possesses the ability to heterotrophically graze on cyanobacteria *Synechococcus* (Jeong et al. 2005, Glibert et al. 2009). Although the clearing rates of *Synechococcus* was not determined in this study, the decrease in percent dominance of OTU 102, identified as a cyanobacteria closely resembling *Synechococcus*, may support this alternate pathway. Much like *K. brevis*, *Synechococcus* is also known to use a variety of DON sources (Glibert et al. 2004, Heil et al. 2007, Sipler 2009); therefore, bacteria may be another vector by which organic nutrients indirectly promote *K. brevis* growth. Although it remains unclear if the *K. brevis* in this study used the TCE DOM directly or indirectly after bacterial remineralization or bactivory, the result of the TCE addition was an overall increase in *K. brevis* abundance.

We found that the *Karenia brevis* community was likely N limited at the start of this experiment and thus responded positively to all bioassays where N (+nitrate, +urea and +TCE) was added. The 3 N treatments differed in the timing and magnitude of the growth response likely due to differences in the uptake pathways as well as differences in overall N additions between the nitrate and urea additions (10 µM nitrate or urea) and the +TCE additions (20 µM DON). Growth was delayed in the +nitrate treatment as compared to the +urea and +TCE treatments. There was no significant increase in cell abundance in the control treatment indicating that N, specifically the nitrate, urea or DON supplied in the TCE, was the catalyst for the observed increase. Nutrient (N:P) gradients observed along the West Florida Shelf are believed to play an important role in determining the phytoplankton community composition (Heil et al. 2007). For example, *K. brevis* is often observed during low N:P or N limited conditions, as opposed to cyanobacteria which prefer a more balanced N:P ratio (Heil et al. 2007).

Karenia brevis growth response

High *Karenia brevis* growth rates (μ, d^{-1}) are not commonly observed in field populations (μ = 0.11 to 0.68 d^{-1}; examples in Loret et al. 2002, Van Dolah et al. 2008, Vargo 2009), which make modeling the growth of *K. brevis* difficult, and require the development of complex ecological models to explain what conditions might allow this 'slow-growing' species to thrive (Bissett et al. 2008). Additionally, traditional sampling techniques poorly resolve dynamics associated with harmful algal bloom (HAB) populations (Stolte & Garcés 2006, Schofield et al. 2008). This study shows that *K. brevis* can double within 24 h on autochthonous DON sources, which simplifies the dynamics required to model red tides.

The growth response observed in the +nitrate treatment differed from that of either the +urea or +TCE treatments in that cell number only significantly increased in abundance between t_{12} and t_{24}, when light was available for photosynthesis (Fig 1). This delay in growth appears to reflect the metabolic cost of reducing the oxidized nitrate and can account for the reduced division rate potential for *Karenia brevis* cultures grown on nitrate. The decrease in overall cell number between t_{12} and t_{24} in the +urea treatment is likely due to N limitation, as the +TCE treatment (which received twice the N addition) achieved a cell concentration that was twice that of the +urea treatment. Based on the initial uptake and growth response of the population to the urea addition, the +urea and +TCE treatments would likely have had similar growth responses if supplied at the same final N concentration. This is noteworthy as urea is an important component of anthropogenic N and represents >50% of the nitrogenous fertilizer used globally (Glibert et al. 2006). Beyond being a potential source of N to coastal systems, increased urea fertilization correlates with an increase in observed HABs (Anderson et al. 2002). The stimulation of *K. brevis* growth by urea could have implications for the effects of agriculturally based eutrophication on coastal *K. brevis* blooms. In addition to urea being directly usable by *K. brevis*, Heil et al. (2007) found that West Florida Shelf cyanobacteria populations, a potential prey source for *K. brevis* (Glibert et al. 2009), responded positively to urea additions presenting another vector by which urea may support *K. brevis* growth.

The observation of rapid N drawdown and growth presented here should be useful to researchers planning experiments in the future. We chose our N additions and timing of sampling with the assumption that *Karenia brevis* was slow growing — based on the literature to date and concern over not making excessive N additions. Based on the growth response presented here and in Ransom Hardison et al. (2012), we suggest future studies account for at least 1.5 to 2 μmol N million *K. brevis* $cells^{-1}$ l^{-1} and a maximum of a doubling of *K. brevis* cells per day.

Chemical characterization: labile vs. refractory compounds

In this study we show that the DOM produced by *Trichodesmium* is a highly labile and likely important source of nutrients to *Karenia brevis* blooms. At least 53% of DON produced by *Trichodesmium* was directly or indirectly available to the *K. brevis* bloom community. This is the first quantitative estimate of the liability of DOM, specifically DON, produced by *Trichodesmium* to co-occurring *K. brevis* communities.

ESI-MS analysis revealed that 27% of all compounds present in the initial +TCE bioassay decreased to below detection within the first 24 h. This is mirrored by the FT-ICR MS analyses, as 23% of all compounds detected under higher resolution also disappeared within the first 24 h. Due to the tight coupling of these analyses, we focused on those compounds that disappeared as the potentially bioavailable compounds that fueled the increase in *Karenia brevis* abundance. Based on those compounds that disappeared within 24 h, there is a distinct preference by the *K. brevis* community for reduced DON and DOP compounds. The most abundant amino acid found to be produced by *Trichodesmium* is glutamate (Capone et al. 1994). While positive identifications of specific compounds can only be made through the addition of standards, a compound with the molecular weight and molecular formula similar to glutamate was observed in the *K. brevis* bloom water and the +TCE treatment at t_0, but had decreased to below detection by t_{24}. Glutamate has H:C and O:C molar ratios of 1.6 and 0.8, respectively, and falls near the protein region of the van Krevelen diagram (Fig. 3). The protein and lipid-like regions of the diagram are often dominated by microbial byproducts (Bhatia et al. 2010) and it is this region (H:C > 1.5 and O:C < 0.8) where a large portion of the potentially bioavailable DON (44%) and DOP (39%) compounds are found.

The number of bioavailable DON compounds is likely an underestimation due to the limitations of molecular formula assignments, the loss in recovery associated with the DOM salt removal and concen-

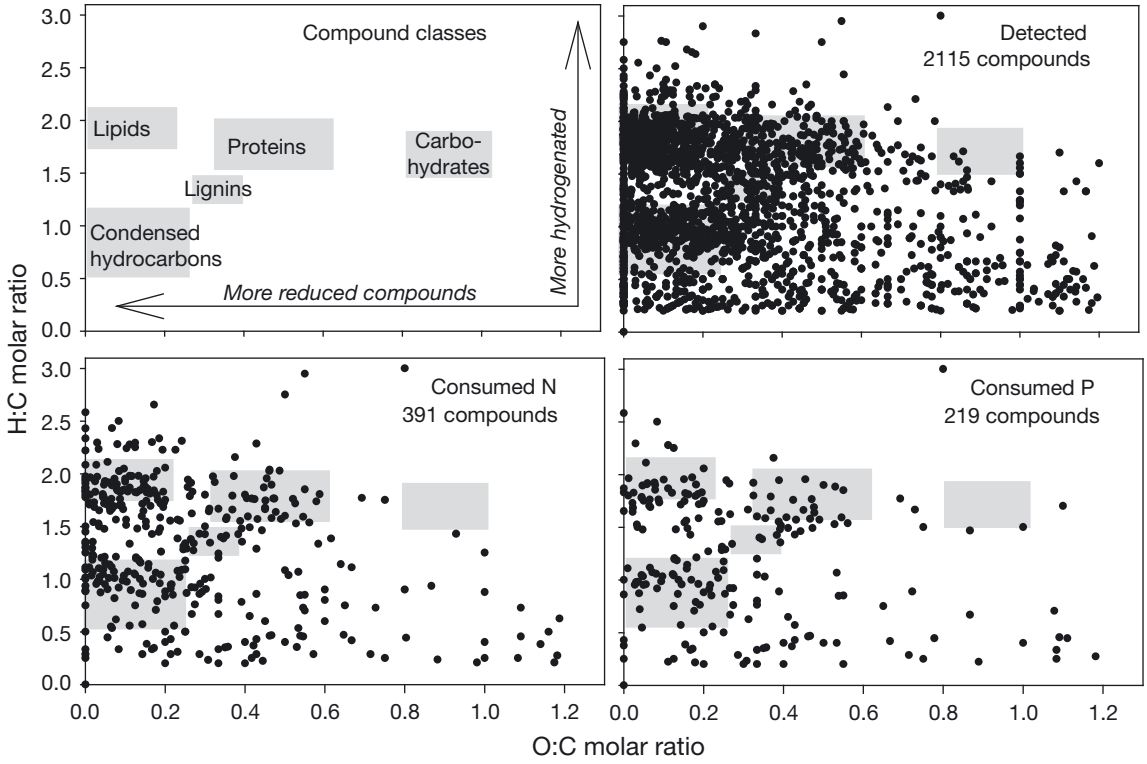

Fig. 3. Van Krevelen diagrams of general compounds classes redrawn from Kim et al. (2003) and Bhatia et al. (2010); all formula assigned for +*Trichodesmium* sp. cellular exudates (+TCE) bioassays, N-containing organic compounds consumed, and P-containing organic compounds consumed

tration method, and the ionization efficiency of individual compounds. There was a 46 to 47% molecular formula assignment efficiency for compounds detected (Table 1). Although the number of high resolution marine DOM studies is still relatively small compared to other fields, most studies on marine DOM report molecular formula assignments of 80% or better (Sleighter & Hatcher 2008, Kujawinski et al. 2009, Kido Soule et al. 2010). The discrepancy in molecular formula assignment efficiency is not due to differences in instrumental or analytical methods, as all FT-ICR MS samples presented here were processed according to accepted methods (methods reported in Bhatia et al. 2010, Kido Soule et al. 2010). The observed differences in molecular formula assignment efficiency may be due, in part, to differences in the extraction method or to the composition of this very biologically influenced sample. Until relatively recently, most DOM extraction for high resolution assessments have used solid phase extraction methods, most notably C18 extractions (Mopper et al. 2007, Dittmar et al. 2008), which recovers 30 to 60% of DOM. The method used in this study has a greater retention of DOM and of larger ions due to the additional ultra filtration step that retained compounds larger than 1000 Da (Simjouw et al. 2005). This is also a very complex sample of microbially produced DOM that may contain large multiply charged ions or compounds that contain elements and metals not included in the parameters investigated.

The observed H:C ratios are slightly higher than are typically found in other marine studies (Fig. 3; examples include Koch et al. 2005, Sleighter & Hatcher 2007). This difference is likely due to the high number of DON compounds observed and the ionization mode. Since the samples investigated in this study came from either a culture or an area of active N fixation, the large number of DON compounds is expected.

Ultra-high resolution FT-ICR MS is a relatively new application to marine DOM. To our knowledge this is the first study using this method to investigate the relationship between harmful algae and DOM. The compounds investigated here represent previously uncharacterized compounds produced by *Trichodesmium* that support the microbial community dominated by *Karenia brevis*.

Bacterial community composition

While the nutrients supplied in the bioassay treatments resulted in an increase in *Karenia brevis* abundance, it also supported a bacterial community. Field observations show that bacterial abundance as non-algal particles negatively correlate with *K. brevis* abundance, indicating that a negative relationship may exist between *K. brevis* and bacterial populations (Schofield et al. 2006). Although several studies have investigated the bacterial species associated with *K. brevis* cultures, the relationship of natural *K. brevis* blooms to their co-occurring bacterial community remains understudied at the community level (Jones et al. 2010, Sipler et al. in press). From those studies that have investigated the bacteria within *K. brevis* blooms, *Alphaproteobacteria* appear to represent the dominant bacterial group (Kodama et al. 2006, Jones et al. 2010, Sipler et al. in press). *K. brevis* abundance also appears to negatively affect several of the GOM bacterial species including several *Cyanobacteria* and *Actinobacterium*-like species (Jones et al. 2010, Sipler et al. in press). Some possible ways that *K. brevis* may affect bacteria include competition for nutrients (Caron 1994), direct phagotrophic grazing (Jeong et al. 2005, Glibert et al. 2009), or negative impacts on the community due to toxin production (Sipler et al. in press). Although brevetoxin concentrations were not measured in this study, N limitation has been shown to increase brevetoxin production within *K. brevis* cultures (Ransom Hardison et al. 2012).

While the total number of bacterial species (OTUs) detected decreased over the first 24 h of the TCE bioassay, some bacterial species were resilient and maintained dominance within the total bacterial population. These resilient OTUs were mostly *Alphaproteobacteria* and may represent natural populations of algicidal bacteria and those bacteria unaffected by *Karenia brevis* and its neurotoxins (Table 2).

CONCLUSIONS

Karenia brevis abundance doubled in the presence of *Trichodesmium*-derived DOM, confirming that *K. brevis* is not only able to directly or indirectly use TCE DON as a nutrient source but that it can grow rapidly on DON substrates. Based on the complete utilization of the DON supplied in the +TCE bioassay, at least 53% of DON produced by *Trichodesmium* is bioavailable to the microbial community dominated by *K. brevis*. The bacterial community also changed throughout the first 24 h, revealing both negative and positive relationships between *K. brevis* and native bacteria species. *Synechococcus*-like species dominance decreased as *K. brevis* abundance increased, supporting the predator–prey (Jeong et al. 2005, Glibert et al. 2009) or bacteriocidal (Sipler et al. in press) hypotheses. Conversely, several Alphaproteobacterial species increased in dominance as *K. brevis* abundance increased, revealing a potential positive or neutral relationship. While these relationships are likely very complex in nature, the community structure is likely significantly influenced by the availability (concentration and composition) of nutrients provided. The DOM supplied in the +TCE bioassays was composed of a very complex suite of compounds dominated by protein and lipid-like compounds closely resembling other observations of microbial byproducts. These TCE protein and lipid-like compounds represented nearly half of all of the DON and DOP compounds consumed during the first 24 h. Nitrate and urea also serve as important potential N sources and *K. brevis* concentrations would have likely doubled in the +urea treatment if more urea had been added. This study shows that *K. brevis* is an opportunistic species that has the ability to take full advantage of a wide range of N sources, including compounds from both anthropogenic (nitrate and urea) and natural (nitrate, urea and DON produced by *Trichodesmium*) sources.

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