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EFFECT OF SALINITY ON DMSP PRODUCTION IN *GAMBIERDISCUS BELIZEANUS* (DINOPHYCEAE)[1]

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Dimethylsulfoniopropionate (DMSP) is produced by many species of marine phytoplankton and has been reported to provide a variety of beneficial functions, including osmoregulation. Dinoflagellates are recognized as major DMSP producers, although accumulation has been shown to be highly variable in this group. We explored the effect of hyposaline transfer in *Gambierdiscus belizeanus* between ecologically relevant salinities (36 and 31) on DMSP accumulation, Chl *a*, cell growth, and cell volume, over 12 d. Our results showed that *G. belizeanus* maintained an intracellular DMSP content of 16.3 pmol·cell^{-1} and concentration of 139 mM in both salinities. Although this intracellular concentration was near the median reported for other dinoflagellates, the cellular content achieved by *G. belizeanus* was the highest reported of any dinoflagellate thus far, owing mainly to its large size. DMSP levels were not significantly affected by salinity treatment, but did change over time during the experiment. Salinity, however, did have a significant effect on the ratio of DMSP:Chl *a*, suggesting that salinity transfer of *G. belizeanus* induced a physiological response other than DMSP adjustment. A survey of DMSP content in a variety of *Gambierdiscus* species and strains revealed relatively high DMSP concentrations (1.0–16.4 pmol·cell^{-1}) as well as high intrageneric and intraspecific variation. We conclude that, although DMSP may not be involved in long-term (3–12 d) osmoregulation in this species, *G. belizeanus* and other *Gambierdiscus* species may be important contributors to DMSP production in tropical benthic microalgal communities due to their large size and high cellular content.

Key index words: **dinoflagellate; DMSP; *Gambierdiscus* spp.; hyposaline transfer; osmolyte; osmoregulation; salinity**

***Abbreviations*: Chl *a*, chlorophyll *a*; CP, ciguatera poisoning; CV, coefficient of variance; DMS, dimethyl sulfide; DMSP, dimethylsulfoniopropionate; $DMSP_i$, intracellular DMSP; $DMSP_p$, particulate DMSP; SD, standard deviation;**

Many marine phytoplankton species are known to produce and accumulate the sulfur-containing compound dimethylsulfoniopropionate (DMSP). DMSP has been associated with a variety of physiological functions in algae including cryoprotection (Kirst et al. 1991, Nishiguchi and Somero 1992, Karsten et al. 1996), scavenging of reactive oxygen species (Sunda et al. 2002, Archer et al. 2010, Husband et al. 2012), and osmoregulation (Dickson et al. 1980, Dickson and Kirst 1986, Van Bergeijk et al. 2003, Lyon et al. 2011, Zhuang et al. 2011, Kettles et al. 2014). As an osmoregulator, DMSP may be preferential to nitrogen-containing solutes such as glycine betaine, since sulfur is ubiquitously present in high concentrations throughout the marine environment while nitrogen is often limiting (Kirst 1996, Keller et al. 1999). The production of DMSP has also been proposed as an overflow mechanism for photosynthetic cells to prevent negative feedbacks on metabolism associated with the build-up of excess sulfur-containing amino acids (Stefels 2000). Several studies have suggested that DMSP and its degradation products may exert antimicrobial (Slezak et al. 1994, Raina et al. 2016) and antiviral properties (Evans et al. 2006, 2007). Furthermore, these compounds are thought to be ecologically important due to their role in grazer deterrence (Wolfe et al. 1997), chemical signaling (Nevitt 2000, Garren et al. 2014, Dove 2015), and in marine microbial food webs (Steinke et al. 2006, Evans et al. 2007, Johnson et al. 2016). In addition to its physiological and ecological functions, DMSP plays an important role in global biogeochemistry as the main precursor of dimethyl sulfide (DMS), the major volatile sulfur compound emitted from the oceans to the atmosphere (Lovelock et al. 1972, Andreae 1990). DMS seems to be a major source of climate-active aerosols under phytoplankton bloom conditions (Quinn et al. 2017) and may exert a cooling effect on the global climate by promoting cloud formation (Charlson et al. 1987).

Although DMSP production is widespread in marine microalgae, it is not always well correlated with traditional estimates of phytoplankton abundance like chlorophyll *a* (Andreae 1990, Kirst et al. 1991, Belviso et al. 1993), which is attributable to interspecific variation in DMSP content. A survey of 123 strains of phytoplankton encompassing several

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taxonomic classes revealed that the most significant contributors to DMSP production are the Dinophyceae (dinoflagellates) and Prymnesiophyceae (mainly coccolithophores; Keller et al. 1989). However, this generalization disregards the substantial variability observed within dinoflagellates where DMSP accumulation spans several orders of magnitude between species (Caruana and Malin 2014). To improve estimates of DMS flux from phytoplankton communities, it is necessary to consider the species composition of microalgal communities, the relative contribution of DMSP by each species, and the environmental and physiological factors that may control or contribute to DMSP production. There have been several investigations of DMSP production by marine dinoflagellates (reviewed by Caruana and Malin 2014), including some of the first reports of DMSP production in *Amphidinium carterae* and *Crypthecodinium cohnii* (Ackman et al. 1966, Ishida 1969) as well as the elucidation of DMSP production pathways by Uchida et al. (1996). More recent work has provided evidence of substantial but variable DMSP production by dinoflagellates in the genus *Symbiodinium*, which exist as symbiotic coral zooxanthellae (Broadbent et al. 2002). Given the extremely high variability of DMSP accumulation in dinoflagellates, Caruana and Malin (2014) sought to explore which characteristics may be associated with variable DMSP production. They found that bioluminescence and plastid type were significantly related to DMSP production but not to geographic origin, the presence of a theca, or with phylogenetic position, depending on the resolution of the taxonomic groupings. Although not quite significant, there was evidence for a potential link between toxin and DMSP production in the observations included in their analysis, which suggests that toxigenic species may be important sources of DMSP in the environment.

Dinoflagellates of the genus *Gambierdiscus* are most commonly found as epiphytes on seagrass, macroalgae, coral, and other benthic substrates in tropical, subtropical, and warm temperate waters throughout the world (Litaker et al. 2010, Parsons et al. 2012, Larsson et al. 2018), including the Caribbean (Ballantine et al. 1985, McMillan et al. 1986, Litaker et al. 2017) and northern Gulf of Mexico (Villareal et al. 2007, Tester et al. 2013). *Gambierdiscus* spp. are among the largest dinoflagellates, with a lenticular shape and diameters typically ranging from 40 to 100 μm (Richlen et al. 2008, Litaker et al. 2009). This genus is recognized as a source of toxigenic secondary metabolites known as ciguatoxins which can be biotransformed and bioaccumulated in marine food webs and cause ciguatera poisoning (CP; Randall 1958, Helfrich and Banner 1963, Yasumoto et al. 1977). Like ciguatoxins, DMSP also originates from basal sources including corals containing zooxanthellae, benthic algae, and phytoplankton, and can be transferred through food webs in reef environments (Dacey et al. 1994, Hill et al. 1995).

Despite numerous studies on DMSP production by dinoflagellates, there is only one report to date on DMSP content in *Gambierdiscus*. Keller et al. (1989) tested one strain of *Gambierdiscus toxicus* (GT200A) which accumulated an intracellular DMSP concentration of 10.1 mM and a cellular content of 160 pg (1.1 pmol) DMSP. In this study, we investigated how cellular DMSP varied with growth in *G. belizeanus*, a toxigenic species of Caribbean origin. Although the ecology, toxin production, and growth of *Gambierdiscus* spp. are a focus of many studies on CP (including in our laboratory), there are few studies that have addressed osmoregulation and the role of organic sulfur compounds in this genus. Therefore, we tested the effect of transfer from high to low salinities on DMSP production in *G. belizeanus*. Additionally, we surveyed the cellular content of DMSP in nine strains of four other *Gambierdiscus* species to explore intrageneric and intraspecific variability of DMSP accumulation. As a key species in epibenthic phytoplankton communities, this information may aid in understanding the production and ecological role of DMSP in this genus.

MATERIALS AND METHODS

Gambierdiscus belizeanus cultures. Keller's (K) Culture Medium (National Center for Marine Algae and Microbiota; NCMA; East Boothbay, ME, USA) was prepared and salinity was adjusted using Instant Ocean sea salt mixture (Spectrum Brands, Blacksburg, VA, USA) and final media was sterilized through a 0.2 μm Isopore membrane filter (MilliporeSigma, Burlington, MA, USA). *G. belizeanus* (CCMP 399) was obtained from NCMA. Batch cultures (560 mL) were acclimated to 36 in a modified K medium ($2 \times NO_3^-$; no NH_4^+, Tris or Cu) as described by Kibler et al. (2012). Cultures were maintained in a Fisher Scientific Isotemp incubator (Hampton, NH, USA) at 25°C under mounted 15-W fluorescent lamp bar (TorchStar, Inc., City of Industry, CA, USA) with an irradiance of 55 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} ($\pm$ 10%) with a light:dark cycle of 12:12 h. Irradiance was measured using a LI-190/R sensor attached to a LI-1400 datalogger (LI-COR, Inc., Lincoln, NE, USA). Cultures were considered acclimated when growth rates were consistent (i.e., varied less than 10%) for at least five successive transfers. Once acclimated, cultures in exponential growth were seeded into 12 experimental batch cultures (50 mL) at a concentration of 200 cells $\cdot$ mL^{-1} for each of two treatments: high salinity (36) and low salinity (31). These salinities are consistent with environmental data from our long-term monitoring sites in the U.S. Virgin Islands and other regions of the Caribbean Sea, from which CCMP399 was reported to be isolated. Furthermore, these salinities fall within optimal salinity ranges for *G. belizeanus* in culture, which have been reported to range from 22.4 to 36.7 (Kibler et al. 2012) and 26.1 to 42.8 (Xu et al. 2016).

Sampling. Samples were taken from each salinity treatment on every third day for the 12 d experimental period. Day 0 measurements were taken in triplicate from the parent culture prior to seeding experimental flasks, while measurements for each subsequent time point were taken in triplicate from each of three sacrificial replicate flasks harvested on

that day. For each flask, triplicate samples were taken for analysis of cell density, cell volume, chlorophyll *a* (Chl *a*), total DMSP concentration, and intracellular DMSP concentration. The general experimental design and sampling scheme are summarized in Figure 1.

Cell concentration, growth rate, and volume estimation. For each culture flask, cell concentrations (cells · mL^{-1}) were determined using a 1 mL gridded Sedgewick Rafter counting chamber (Wildco, Yullee, FL, USA). Growth rates were estimated using the equation $\mu = \frac{\ln(C_2/C_1)}{t_2 - t_1}$ where C_2 and C_1 represent the average cell concentrations of the triplicate flasks harvested for a given salinity treatment at each time point and $t_2 - t_1$ represents the amount of time (in days) between time points. A sample of each culture was preserved in 5% formalin which maintains morphological characteristics of cells (M. Richlen, pers. comm.). Cell volume was subsequently determined in formalin-preserved samples by measuring cell dimensions under light microscopy (200 × magnification). *Gambierdiscus* spp. are lenticular in shape, so cell volume was approximated as the volume of an ellipsoid, $V = \frac{4}{3}\pi\, abc$ where *a* and *b* represent the radii of the major and minor axes of the apical plate, respectively, and *c* represents the radius of the anterior–posterior axis (Olver et al. 2010).

Chl *a* extraction and determination. Chl *a* was extracted in 90% acetone as outlined in EPA Method 445.0 (Arar & Collins 1997) and analyzed by the non-acidification method described by Welschmeyer (1994). Samples were then analyzed on a Trilogy fluorometer (Turner Designs, San Jose, CA, USA) fitted with a Chl *a* non-acidified module. Standards of Chl *a* (from *Anacystis nidulans,* Sigma C6144, Sigma-Aldrich, St. Louis, MO, USA) were used to calibrate the fluorometer and instrument performance was validated with a solid secondary standard prior to sample analysis. Chl *a* samples were taken in triplicate from each flask, duplicate aliquots made from each sample extraction, and two analytical replicates were performed for each aliquot.

DMSP sample and standard preparation. Total and particulate DMSP samples were prepared in triplicate for each flask while blank samples for each fraction were prepared in duplicate. Particulate DMSP ($DMSP_p$) represented the DMSP associated with cells retained on the filter only, and intracellular DMSP ($DMSP_i$) contents and concentrations were calculated by normalizing $DMSP_p$ to the volume and number of filtered cells, respectively. Intracellular DMS was expected to be negligible due to its reported high permeability through cell membranes, which limits the amount retained within algal cells (Spiese et al. 2016). The intracellular $DMSP_i$ was also normalized to intracellular Chl *a* to determine the ratio of DMSP: Chl *a*, which has been used previously to estimate contribution of DMSP to phytoplankton biomass (Kiene et al. 2000, Simo et al. 2002). The total DMSP and DMS ($DMSP_t$ + DMS) represented the total DMSP in the culture, either associated with the cells or in the media, along with any DMS present in the culture since samples were not acidified during preparation. The difference between total $DMSP_t$ + DMS and $DMSP_p$ was calculated to estimate dissolved DMSP and DMS in the media ($DMSP_d$ + DMS). The pool of $DMSP_d$ + DMS was found to be negligible relative to $DMSP_p$ which was expected given that cultures were open to air and were non-axenic.

Total DMSP samples were prepared by transferring 1 mL of a well-mixed culture into a 14 mL glass vial with 1 mL of strong base (5 M NaOH) to hydrolyze DMSP to gaseous DMS. $DMSP_p$ was prepared by filtering a 1 mL aliquot of each well-mixed experimental culture through a glass microfiber filter with a nominal pore size of 0.7 µm (Whatman GF/F, GE Healthcare Life Sciences, Pittsburgh, PA, USA) under low vacuum (−100 mm Hg). Filters, which retained approximately 0.3 mL liquid, were then transferred into glass vials containing 0.7 mL sterile media of the same salinity to ensure consistent sample volumes between total and intracellular samples. An equal volume (1 mL) of strong base (5 M NaOH) was then added to each sample vial to hydrolyze DMSP to gaseous DMS and each vial. On each sampling day, a dilution series of DMSP·HCl (Bioviotica, Dransfeld, Germany) standards were prepared in sterile media and hydrolyzed as described above. All samples and standards were sealed immediately following addition of base and allowed to hydrolyze for at least 24 h prior to measurement to ensure thorough cleavage of any DMSP in the sample to DMS (White 1982).

Gas chromatography analysis. DMSP standards and samples were analyzed by direct injection of headspace gas on a Shimadzu GC-2014 gas chromatograph (Kyoto, Japan) fitted with a 6′ × 1/8″ diameter FEP Teflon column filled with Chromosil 330 (Supelco, Inc., Bellefonte, PA, USA) and coupled with a flame-photometric detector. The temperature of the injection port and detector was maintained at 175°C and the column at 60°C. The injection port pressure was held at 17.5 psi and $He_{(g)}$ carrier flow was maintained at 20 mL · min^{-1}.

DMSP concentrations were determined by calculating the square root of DMS peak area and interpolation from the corresponding DMSP·HCl standard curve. Standard curves were considered suitable for interpolation when the coefficient of determination (R^2) equaled 0.99 or greater. For each sample vial prepared, analytical measurements were repeated in triplicate.

Interspecific and intraspecific DMSP content. In addition to the experiments with *Gambierdiscus belizeanus* described above,

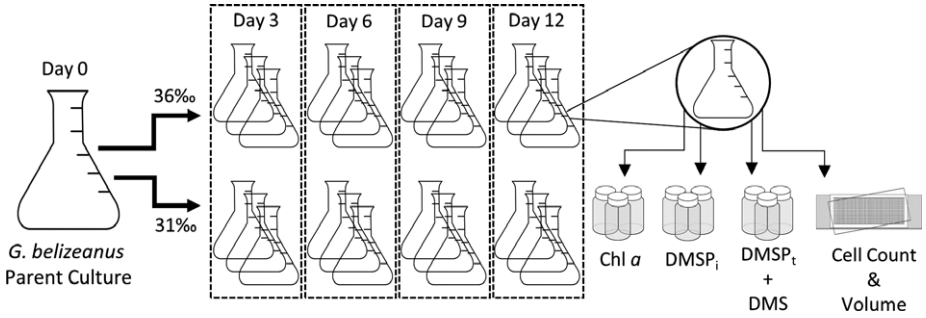

FIG. 1. Diagram showing experimental design of *Gambierdiscus belizeanus* salinity transfer experiment. Parent culture of *G. belizeanus* was acclimated to 36 salinity medium and used to seed experimental flasks at salinities of either 36 or 31. Triplicate flasks from each salinity treatment were sampled every 3 d for 12 d. From each flask, samples were taken in triplicate to determine cell concentration, cell volume, Chl *a*, $DMSP_i$, and $DMSP_t$ + DMS. Day 0 samples were taken directly from the *G. belizeanus* parent culture.

DMSP content per cell was determined for additional strains of *Gambierdiscus* spp. representing the species *G. caribaeus, G. carolinianus* (CCMP 3144; NCMA), *Gambierdiscus* ribotype 2, and *G. silvae*. These cultures were maintained in 35 modified K medium (no Tris or Si) at 25°C and 70 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} ($\pm$ 10%) with a light:dark cycle of 12:12 h. *Gambierdiscus* spp. cells were harvested during exponential growth and triplicate $DMSP_p$ samples were prepared as described above but were filtered through a glass fiber filter with a nominal pore size of 1 μm (Pall A/E, Pall Corporation, Port Washington, NY, USA). While the use of a single filter types is recommended when possible, the effect of pore size (0.7 μm for GF/F and 1.0 μm for A/E glass fiber filters) was confirmed to have no effect on $DMSP_P$ measurements in *Gambierdiscus* spp. (data not shown). Samples and standards were analyzed by gas chromatography as previously described. Cell counts were determined by microscopy as described above to allow calculation of $DMSP_i$ content per cell.

QA/QC and data analysis. Quality of cell counts, cell size measurements, Chl *a* and DMSP data were assured by calculating the coefficient of variation (CV) for analytical and technical replicates, where $\%CV = \frac{SD}{mean} \times 100$ where SD = standard deviation. Mean values were accepted if CV was less than or equal to 20%; for those values where CV exceeded 20%, either the outliers or the entire replicate was omitted from statistical analysis.

The Shapiro–Wilk normality test was used to confirm that data followed a normal distribution prior to parametric analysis. A one-sample *t*-test was used to compare growth rates observed throughout the 12 d experimental period in both salinity treatments with the growth rate attained by acclimated cultures. Ordinary two-way ANOVAs were used to investigate the effect of both salinity and time on *Gambierdiscus belizeanus* growth rates, cell volumes, Chl *a*, DMSP*i* contents and concentrations, and DMSP:Chl *a* throughout the experiment. Null hypotheses were rejected at a significance level of $P = 0.05$. Post-hoc analysis of significant findings was conducted using Sidak's multiple comparisons tests, which compares means among a specific group of observations (e.g., different time points within a salinity treatment or different salinity treatments at a given time point). All analyses were carried out in GraphPad Prism v7.04 (La Jolla, CA, USA).

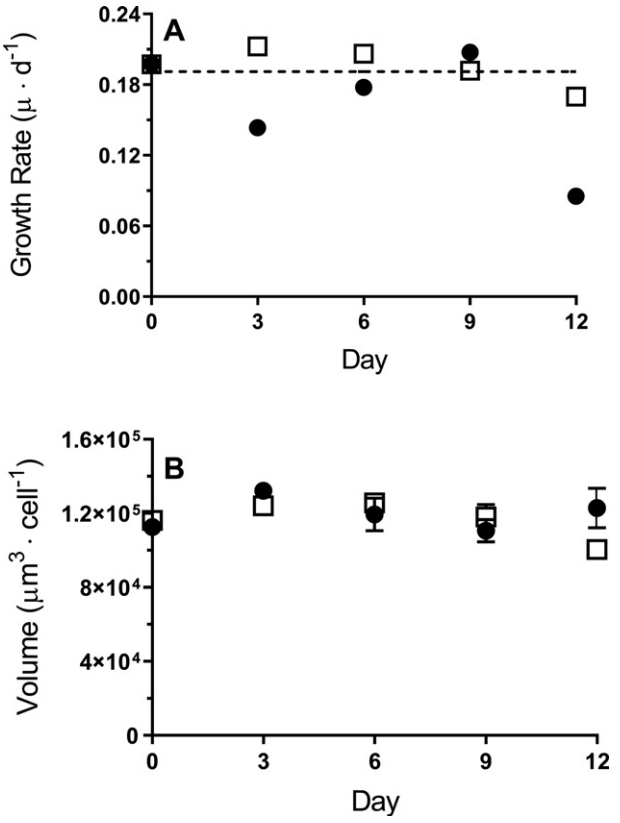

FIG. 2. Growth rate (A) and cell volumes (B) of *Gambierdiscus belizeanus* throughout the experiment in low-salinity (open squares) and high-salinity (filled circles) treatments. The black dotted line in (A) shows the acclimated growth rate of the *G. belizeanus* parent culture prior to the start of the experiment; growth rates did not significantly differ with time (two-way ANOVA, $F_{4,8} = 1.56$, $P = 0.27$) or salinity treatment (two-way ANOVA, $F_{2,8} = 2.12$, $P = 0.18$) throughout the experiment. Contrarily, cell volumes did significantly change over time (two-way ANOVA, $F_{4,16} = 7.02$, $P < 0.01$) but were not dependent on salinity (two-way ANOVA, $F_{1,16} = 0.96$, $P = 0.34$). Error bars in (B) show standard deviation.

RESULTS

Growth rate of Gambierdiscus belizeanus. The parent culture of *G. belizeanus* acclimated to 36 media maintained an average exponential growth rate of 0.19 $\cdot$ d^{-1} (CV = 5.0%). The rate observed in our study was slightly lower than previously reported for this species (Kibler et al. 2012, Xu et al. 2016), possibly due to lower temperature and irradiance selections. Growth rates in the acclimated parent culture and throughout the experiment are shown in Figure 2A. No significant difference in growth rates was found with time (two-way ANOVA, $F_{4,8} = 1.56$, $P = 0.27$) or salinity treatment (two-way ANOVA, $F_{2,8} = 2.12$, $P = 0.18$). Growth rates calculated throughout the experiment in both treatments were not significantly different from the average growth rate of the acclimated culture (36 treatment: one-sample *t*-test, $t_4 = 1.29$, $P = 0.26$; 31 treatment: one-sample *t*-test, $t_4 = 0.62$, $P = 0.57$), which suggests that cultures remained in exponential growth throughout the experiment in both salinities. These findings concurred with Kibler et al. (2012) where *G. belizeanus* growth rates were unaffected by rapid or gradual salinity changes when the final salinity was within the optimal range (22.4–36.7).

Cell size and volume of Gambierdiscus belizeanus. The size of *Gambierdiscus belizeanus* in this study ranged from 50 to 85 μm and displayed a mean $\pm$ SD transdiameter of 67.3 $\pm$ 5.8 μm (CV = 8.7%) throughout the experiment. These cell sizes were similar to those previously observed in *G. belizeanus* (CCMP 399), which reportedly range from 51 to 79 μm (Faust 1995, Richlen et al. 2008).

The cell volume of Gambierdiscus *belizeanus* was similar in both salinity treatments and averaged 1.2 $\times$ 10^5 μm^3 $\cdot$ cell^{-1} (SD = 0.10 $\times$ 10^5; CV = 8.7%) throughout the experiment (Fig. 2B). Salinity was not a significant determinant of cell volume in *G. belizeanus* (two-way ANOVA, $F_{1,16} = 0.96$, $P = 0.34$). However, cell volumes did change significantly over time

(two-way ANOVA, $F_{4,16} = 7.02$, $P < 0.01$). Post-hoc analysis showed that the significant changes over time in the 31 salinity treatment were driven by the decreased cell volumes on day 12, but in the 36 salinity treatment the significant difference only occurred between days 3 and 6. Finally, the interaction of the salinity and time factors had a significant effect on cell volumes (two-way ANOVA, $F_{4,16} = 6.07$, $P < 0.01$) which indicates that, although mean cell volume was similar in both treatments, the patterns of change in cell volume over time differed between salinity treatments.

Chlorophyll content. The average ± SD cellular content of Chl *a* in *Gambierdiscus belizeanus* was 0.16 ± 0.02 ng Chl *a* · cell^{-1} (CV = 10.1%) throughout the experiment in both salinities (Fig. 3A). Contents were significantly affected by salinity, time, and the interaction of these two factors (two-way ANOVA; $F_{1,16} = 9.75$, $F_{4,16} = 11.09$, $F_{4,16} = 6.13$, respectively; $P < 0.01$ for all). Significant differences over time resulted from the increase in Chl *a* content on day 12 while the significant difference between salinity treatments only occurred in the initial measurement on day 0.

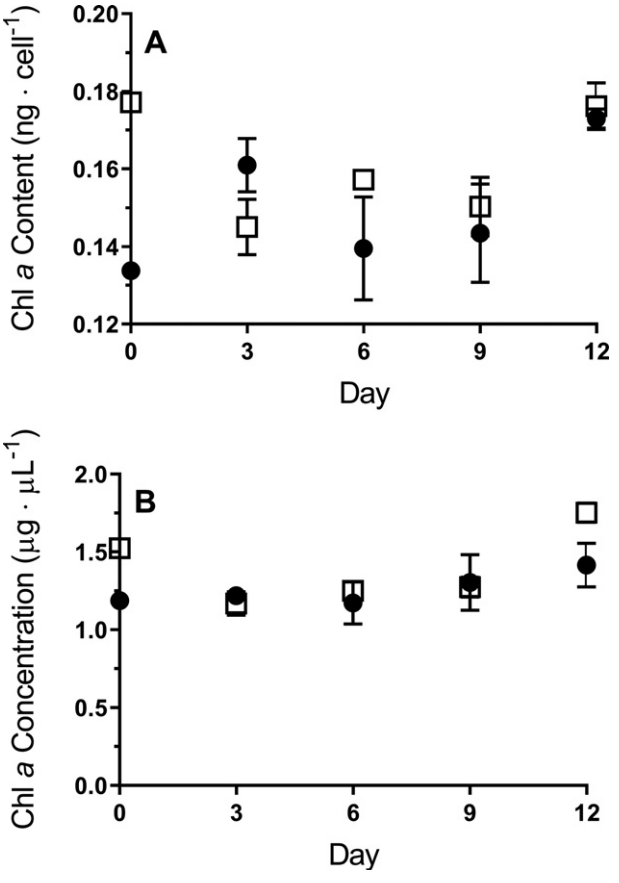

FIG. 3. Chlorophyll *a* content per cell (A) and intracellular concentration (B) in *Gambierdiscus belizeanus* in both low-salinity (open squares) and high-salinity (filled circles) treatments. Chl *a* values were significantly affected by salinity (content: two-way ANOVA, $F_{1,16} = 9.75$, $P < 0.01$; concentration: two-way ANOVA, $F_{1,16} = 9.07$, $P < 0.01$) and time (content: two-way ANOVA, $F_{4,16} = 11.09$, $P < 0.01$; concentration: two-way ANOVA, $F_{4,16} = 13.77$, $P < 0.001$). Error bars show standard deviation.

Intracellular concentrations of Chl *a* showed an average ± SD of 1.33 ± 0.19 µg Chl *a* · µL^{-1} (CV = 14.1%; Fig. 3B). Concentrations generally increased over time throughout the experiment in both treatments and, like Chl *a* content, differed significantly depending on salinity (two-way ANOVA, $F_{1,16} = 9.07$, $P < 0.01$), time (two-way ANOVA, $F_{4,16} = 13.77$, $P < 0.001$), and the interaction of these factors (two-way ANOVA, $F_{4,16} = 3.98$, $P < 0.05$). Post-hoc analysis revealed that changes in Chl *a* concentration were only significant in the 31 salinity treatment and were driven by the relatively high concentration in this treatment on day 12. Similarly, differences between the two salinity treatments were also only significant on day 12 of the experiment.

DMSP content. The mean ± SD cellular content of $DMSP_i$ in *Gambierdiscus belizeanus* throughout this study across salinity treatments was 16.4 ± 1.3 pmol · cell^{-1} (CV = 7.8%; Fig. 4A). *Gambierdiscus belizeanus* maintained a mean ± SD $DMSP_i$ concentration of 139 ± 18.9 mM (CV = 13.6%) throughout the course of both salinity treatments (Fig. 4B). We conducted a survey of $DMSP_i$ content per cell in nine *Gambierdiscus* strains comprising five species (including *G. belizeanus*), which revealed high per-cell contents (1.02–16.4 pmol · cell^{-1}) as well as high variability between and within species (Table 1). Intraspecific variation was as much as 40% in four strains of *G. silvae* and 99% in two species of *Gambierdiscus* ribotype 2. Using the data from all the strains we tested and the value reported for *G. toxicus* by Keller et al. (1989), the overall variability in $DMSP_i$ content in the surveyed *Gambierdiscus* species was 72%.

We did not observe significant changes in $DMSP_i$ cellular contents (pmol DMSP · cell^{-1}) in *Gambierdiscus belizeanus* upon adjustment of media salinity (two-way ANOVA, $F_{1,16} = 0.11$, $P = 0.74$) but time was a significant factor affecting $DMSP_i$ contents (two-way ANOVA, $F_{4,16} = 4.04$, $P < 0.05$; Fig. 4A). Post-hoc testing showed that differences over time were only significant in the 36 salinity treatment and were driven by the relatively high cellular content on day 9. The interaction of time and salinity was not significant (two-way ANOVA, $F_{4,16} = 2.77$, $P = 0.06$), indicating that $DMSP_i$ contents showed similar patterns of change over time in both treatments.

Like the responses observed for cellular contents, $DMSP_i$ concentrations (mM) were not affected by salinity (two-way ANOVA, $F_{1,16} = 0.10$, $P = 0.75$) but were significantly changed over time (two-way ANOVA, $F_{4,16} = 7.96$, $P < 0.01$) in *Gambierdiscus belizeanus* cultures (Fig. 4B). Post-hoc analysis showed that changes in $DMSP_i$ over time were driven by the relatively high concentrations on day 9 in the 36 salinity treatment and on day 12 in the 31 salinity

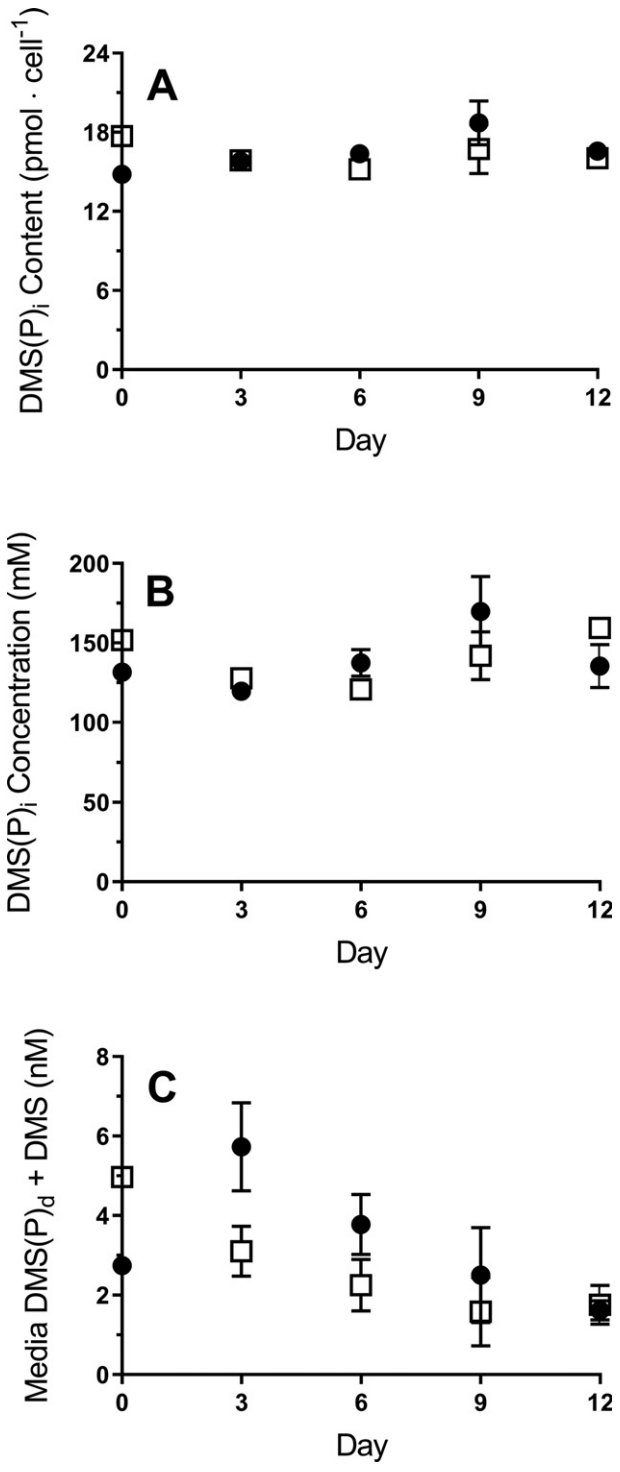

FIG. 4. Intracellular $DMSP_i$ content per cell (A) and concentration (B) in *Gambierdiscus belizeanus* and dissolved $DMSP_d$ + DMS in the media (C) in low-salinity (open squares) and high-salinity (filled circles) treatments. Although time was a significant factor affecting $DMSP_i$ content (two-way ANOVA, $F_{4,16} = 4.04$, $P < 0.05$), concentration (two-way ANOVA, $F_{4,16} = 7.96$, $P < 0.01$), and $DMSP_d$ + DMS (two-way ANOVA, $F_{4,16} = 11.36$, $P < 0.001$), salinity did not significantly affect these $DMSP_i$ content (two-way ANOVA, $F_{1,16} = 0.11$, $P = 0.74$), concentration (two-way ANOVA, $F_{1,16} = 0.10$, $P = 0.75$), or $DMSP_d$ + DMS (two-way ANOVA, $F_{1,16} = 2.47$, $P = 0.13$). Error bars show standard deviation.

TABLE 1. DMSP content (pmol · $cell^{-1}$; average ± SD) by species and strain for *Gambierdiscus* strains tested in the present study and that of *G. toxicus* reported by Keller et al. (1989). Reported transdiameter ranges and average cell volumes are listed for each species, where available; size estimates for *G. toxicus* are not included since this strain (no longer available) likely represents a different species.

Species	Strain	DMSP content (pmol · $cell^{-1}$)	Transdiameter (μm)
G. belizeanus	CCMP 399	16.4 ± 1.3*	51–83[b,c]
G. caribaeus	1604 BP-15	7.8 ± 1.3	70–94[d]
G. carolinianus	CCMP 3144	1.0 ± 0.5	76–103[d]
Gambierdiscus ribotype 2	1509 BP-2	10.7 ± 1.4	–
Gambierdiscus ribotype 2	1508 BP-15	1.9 ± 0.6	–
G. silvae	1602 FC-08	11.9 ± 1.3	55–73[e]
G. silvae	1505 BB-06	8.3 ± 0.6	55–73[e]
G. silvae	1602 BP-05	5.8 ± 2.1	55–73[e]
G. silvae	1510 SH-10	4.9 ± 0.3	55–73[e]
G. toxicus	GT200A	1.2 ± n/a[a]	–

References: (a) Keller et al. (1989); (b) Richlen et al. (2008); (c) Present study; (d) Litaker et al. (2009); (e) Fraga and Rodríguez (2014). *Reported DMSP content for *G. belizeanus* represents mean ± SD throughout the experiment across both 36 and 31 salinity treatments; culture media used in *G. belizeanus* salinity transfer experiments was prepared following Kibler et al. (2012).

treatment. The interaction of time and salinity was a significant factor affecting $DMSP_i$ in Gambierdiscus *belizeanus* (two-way ANOVA, $F_{4,16} = 5.29$, $P < 0.01$), which suggests that overall pattern of change over time differed between salinity treatments.

The average ± SD concentration of DMSP in the media ("$DMSP_d$ + DMS") in the *Gambierdiscus belizeanus* cultures was 2.99 ± 1.42 nM (CV = 47.4%) and generally decreased over time in both salinity treatments (Fig. 4C). We found that differences in mean $DMSP_d$ + DMS were not significant between salinities (two-way ANOVA, $F_{1,16} = 2.47$, $P = 0.13$) but were significant over time (two-way ANOVA, $F_{4,16} = 11.36$, $P < 0.001$). Significant differences over time were driven by the high $DMSP_d$ + DMS on day 3 in the 36 salinity treatment and by day 1 in the lower 31 salinity treatment. Furthermore, the interaction of time and salinity significantly affected $DMSP_d$ + DMS values (two-way ANOVA, $F_{4,16} = 4.54$, $P < 0.05$), which was observable between days 0 and 3 of the experiment, where $DMSP_d$ + DMS shows an initial increase in the high 36 salinity treatment but decreases initially in the 31 salinity treatment.

The ratio of DMSP:Chl *a* content ranged from 88 to 134 nmol DMSP · μg Chl a^{-1} with a mean ± SD of 106 ± 12.9 (CV = 12.2%; Fig. 5), which is on par with previous reports that Chl *a*-normalized DMSP values in dinoflagellates range from 33 to 124 nmol DMSP · μg Chl a^{-1} (Keller and Korjeff-Bellows 1996). In *Gambierdiscus belizeanus*, analysis revealed that DMSP:Chl *a* was significantly affected by time

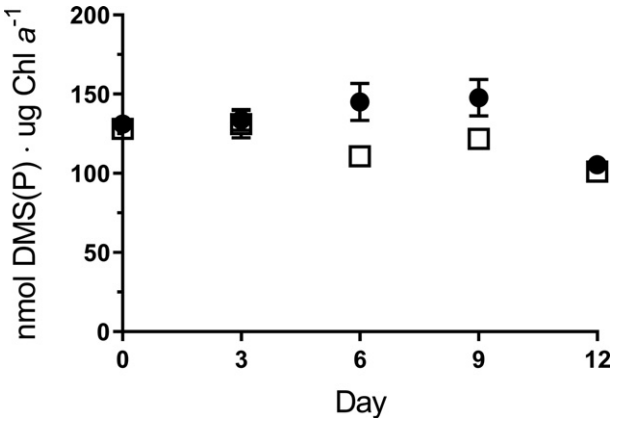

FIG. 5. Chl *a* normalized intracellular DMSP values over time in low-salinity (open squares) and high-salinity (filled circles) treatments. Both time (two-way ANOVA, $F_{1,16} = 20.40$, $P < 0.001$) and salinity (two-way ANOVA, $F_{4,16} = 18.44$, $P < 0.001$) were significant factors affecting DMSPi: Chl *a* ratios throughout the experiment. Error bars represent standard deviation.

(two-way ANOVA, $F_{1,16} = 20.40$, $P < 0.001$), salinity (two-way ANOVA, $F_{4,16} = 18.44$, $P < 0.001$), and the interaction of these factors (two-way ANOVA, $F_{4,16} = 5.81$, $P < 0.01$), which is evident by the differing patterns over time in either salinity treatment. In both salinity treatments, the significant changes in DMSP:Chl *a* ratio are driven by the relatively low values observed on day 12 of the experiment, as revealed by post-hoc analysis. Between salinity treatments, significant differences were found between DMSP:Chl *a* values on days 6 and 9.

DISCUSSION

We found that *Gambierdiscus belizeanus* attains relatively high $DMSP_i$ concentrations of 139 ± 18.9 mM (CV = 13.6%) and cellular contents of 16.4 ± 1.3 pmol · cell^{-1} (CV = 7.8%) under the conditions tested in this experiment. This extent of DMSP accumulation is so far unrecognized in this genus and represents the highest per-cell content of DMSP of any dinoflagellate reported thus far. A review of dinoflagellate DMSP accumulation by Caruana and Malin (2014) reported that intracellular concentrations of DMSP in dinoflagellates range from 0.034 to 7,590 mM ($n = 94$), although values in excess of 1,000 mM are questionable because they exceed the osmolarity of seawater. The concentrations observed in *G. belizeanus* in this study were near the median of those reported by Caruana and Malin (2014) and indicate that DMSP is likely a medium to minor osmolyte in the species, according to their proposed classification of the potential osmotic role of DMSP as it relates to intracellular concentration. The relatively high DMSP concentrations observed in this and other studies support the proposition that DMSP acts as a "constitutive compatible solute" that offers protective functions to the cell while not interfering with normal metabolism, unlike inorganic ions which can elicit cytotoxic effects when present at high levels (Kirst 1996, Stefels 2000). The high cell contents observed in *G. belizeanus* in this study are a result of the relatively high $DMSP_i$ concentration (139 ± 18.9 mM) and the exceptionally large biovolume (118,787 μm^3 · cell^{-1}) of the cells. Our finding contrasts somewhat with previous works which have led to the generalization that while larger cells tend to attain higher $DMSP_i$ contents · cell^{-1}, they have lower DMSP concentrations compared to smaller species (Keller 1989, Keller et al. 1989). High DMSP production is typically attributed to planktonic, bloom-forming species (e.g., coccolithophorids) whose abundance shows substantial variation both spatially and temporally. As such, atmospheric DMS flux tends to be greatest in high latitude regions and shows substantial seasonality, with greater production during the summer months (Lana et al. 2011). In the Caribbean and other tropical and subtropical regions, on the other hand, DMS flux is generally low year-round with a pulse of moderate productivity during the late fall (Lana et al. 2011), although there exist data gaps in these areas throughout much of the year. Unlike coccolithophores and other bloom-forming species associated with high DMSP production, *Gambierdiscus* spp. are widely distributed throughout tropical and subtropical regions worldwide and exhibit relatively consistent abundances year-round. Therefore, given the high cellular $DMSP_i$ content observed in *G. belizeanus* in this study, we believe that this genus may be seasonally important contributors to DMSP production in tropical benthic microalgal communities where they occur. However, further work on the community composition and spatiotemporal dynamics of DMSP production by different benthic microalgal groups is needed to clarify the role and relative importance of *Gambierdiscus* spp. throughout the Caribbean and Eastern Atlantic.

To our knowledge, the production of DMSP has been studied in only one species of *Gambierdiscus* thus far. In Keller et al.'s (1989) survey of phytoplankton, *Gambierdiscus toxicus* reportedly accumulated an intracellular concentration of 10 mM and a cellular content of 1.19 pmol · cell^{-1}, both of which are an order of magnitude lower than values observed in *G. belizeanus* in the present study. It should be noted that the genus *Gambierdiscus* was initially thought to contain only a circumtropically distributed *G. toxicus*, originally described by Adachi and Fukuyo 1979 is referenced here for the original description of *G. toxicus*, but is now known to contain several genetically, morphologically, and geographically distinct species. The strain of *Gambierdiscus* observed by Keller et al. (1989; Bigelow strain GT200A) was reportedly isolated from Knights Key, FL (Bomber et al. 1988), far outside of the currently recognized natural range of *G. toxicus* throughout the Pacific and Indian Oceans (Litaker et al. 2009). Although *Gambierdiscus* strain GT200A is no longer available in culture collections and

genetic identification was not possible, it is likely that the strain studied by Keller et al. (1989) was actually *G. belizeanus*, *G. carolinianus, G. carpenteri, or G. caribaeus*, based on reported phylogeographic distributions.

In our survey of various *Gambierdiscus* strains, we found relatively high $DMSP_i$ per cell in all strains tested (1.0–16.4 pmol · $cell^{-1}$) as well as high interspecific and intraspecific variability in DMSP accumulation. This latter finding is not surprising considering the variability already known to be present in dinoflagellates (Keller et al. 1989, Steinke et al. 2011, Caruana and Malin 2014). Differences in cell sizes between the *Gambierdiscus* species used in this study show substantial overlap (Table 1; Richlen et al. 2008, Litaker et al. 2009, Fraga and Rodríguez 2014) and therefore do not account for the large variability in per cell $DMSP_i$ contents either between or within species and suggest that intracellular DMSP concentrations (not measured) would show similar variation. The observed variation in DMSP accumulation within the genus *Gambierdiscus* suggests that both species composition and strain composition should be considered in estimates of community DMSP production. The production of DMSP by microalgae has been shown to be affected by light (Karsten et al. 1990, Stefels and Van Leeuwe 1998, Kettles et al. 2014), temperature (Spielmeyer and Pohnert 2012), nutrient limitation (Gröne and Kirst 1992, Kettles et al. 2014), and physiological condition or growth phase (Stefels and Van Boekel 1993, Zhuang et al. 2011, Gao et al. 2012). Therefore, it is important that in experiments assessing interspecific and intraspecific variability of DMSP that these conditions are standardized as much as possible. In the present study, we opted to harvest all samples across experiments in exponential growth which reduced the likelihood of under or overestimating microalgal DMSP production due to microbial production or degradation that can occur as cultures enter stationary and senescent phases.

Despite the high cellular contents and intracellular DMSP concentrations observed in *Gambierdiscus belizeanus*, these values were not significantly adjusted in response to transfer between the salinity treatments tested in this study. Therefore, although DMSP may serve as an osmolyte, it does not seem to play a significant role in the osmotic acclimation of *G. belizeanus* over the 3–12 d timescale of our experiment. Some phytoplankton adjust their cell volumes in response to salinity changes as an initial mechanism for osmotic adjustment (Dickson and Kirst 1986), allowing water to flow into or out of the cell as the balance of osmotic potential is restored across the cell wall. If this process were occurring, we would expect per cell content of $DMSP_i$ to remain constant while both cell volume and $DMSP_i$ concentration change. However, the lack of significant changes in cell volume or intracellular concentration observed in this study indicate that volume adjustment is not an important means of osmoregulation in *G. belizeanus*. Although salinity treatment did not significantly affect DMSP accumulation in *G. belizeanus*, we did see significant differences in $DMSP_i$ concentrations and per cell contents over time in both treatments. Furthermore, we found a significant interaction of time and salinity (i.e., the patterns of change over time significantly differed between salinity treatments for $DMSP_i$ and $DMSP_d$ + DMS). We believe that the observed changes in DMSP result from changing conditions associated with the development of the batch culture through the exponential growth phase and that the response over time may be modulated somewhat by external salinity. Some previous studies have shown that the release of DMSP and DMS from algal cells is greatest during the late stationary and senescent growth phases (Stefels and Van Boekel 1993, Zhuang et al. 2011, Gao et al. 2012). However, in this study, non-axenic cultures of *Gambierdiscus* spp. were harvested in exponential growth to reduce the influence of microbial communities which become prominent in later growth stages and may significantly alter and potentially lead to under or overestimates of microalgal DMSP production.

Results of previous studies investigating the importance of DMSP for osmoregulation in marine algae are ambiguous. Experiments with *Phaeocystis* sp. have shown that, although the species accumulates comparable intracellular concentrations to those observed in this study during stationary growth (Stefels and Van Boekel 1993), it exhibits no drastic change in DMSP levels upon salinity change (Stefels et al. 1996). However, transfer to hyposaline media (as was done in the present study) has been shown to cause a rapid release of DMSP in other microalgal and macroalgal species (Dickson et al. 1980, Dickson and Kirst 1986, Van Bergeijk et al. 2003), which suggests that DMSP does play an osmoregulatory role in some organisms. In these previous studies, the magnitude of salinity change was larger than the ecologically relevant range employed in this study, which may not have been large enough to induce alterations in cellular DMSP in *Gambierdiscus belizeanus*. Previous studies may overstate the relevance of DMSP as an osmoregulator rather than an osmolyte, depending on the range of environmental conditions to which the study organisms are naturally exposed compared to the ranges selected in laboratory experiments.

It has been previously proposed that, while DMSP likely contributes substantially to the osmotic potential, the relatively slow response of the solute to changes in salinity (relative to inorganic ions) limits its utility as an osmolyte but may offer a buffering effect to rapid salinity changes and contribute secondarily to more "fine" adjustments of osmolarity (Dickson and Kirst 1986). However, the lack of a

movement to a new DMSP-baseline over the 12 d observation period suggests that DMSP may also not be important for long-term acclimation to environmentally relevant salinity changes in *Gambierdiscus belizeanus*. Certainly, it is possible that the experimental design employed here did not have a high enough time resolution to detect a significant effect on DMSP adjustment in *G. belizeanus*. Previous investigations have shown that, while DMSP levels may change over several days in some species (Van Bergeijk et al. 2003), adjustment of DMSP can occur within hours of salinity shock in others (Dickson et al. 1980, Dickson and Kirst 1986), a much smaller time frame than the 3 d sampling intervals used in the present study.

Chlorophyll *a* was measured and DMSP:Chl *a* ratios estimated to detect potential physiological changes in *Gambierdiscus belizeanus* throughout the experiment and to investigate the relationship between Chl *a* and DMSP production. Unlike DMSP, Chl *a* values were significantly affected by both time and salinity in *G. belizeanus*. This indicates that *G. belizeanus* physiologically adjusts to transfer between salinities of 36 and 31 by altering the abundance of some cellular constituents (e.g., Chl *a*) but not others (e.g., DMSP). The DMSP:Chl *a* ratio has been used to estimate the relative contribution of DMSP to phytoplankton biomass (Kiene et al. 2000, Simo et al. 2002) based on empirical relationships between phytoplankton Chl *a*, carbon, and sulfur. However, we found that DMSP:Chl *a* varies in culture suggesting that ratios of cellular constituents may change with physiological condition, as has been previously described of C:Chl *a* in response to temperature, nutrient, and light status (Geider 1987, Halsey and Jones 2015). Such ratios should therefore be used cautiously when estimating the contribution of DMSP to cellular carbon and sulfur.

Like DMSP accumulation, toxin production by *Gambierdiscus* spp. is highly variable between strains (Holmes et al. 1991, Roeder et al. 2010, Litaker et al. 2017) and may be altered by environmental conditions (Bomber et al. 1988, Roeder et al. 2010), as has also been shown for other toxigenic dinoflagellates like *Karenia brevis* (Errera and Campbell 2011). Furthermore, Caruana and Malin (2014) reported a potential relationship between DMSP production and toxicity in marine dinoflagellates. Given the known variation in toxin production and the accumulation of DMSP observed in this study, it is plausible that there exists a link between toxin and DMSP dynamics in the genus *Gambierdiscus*. Furthermore, the purported role of DMSP in grazer deterrence (Wolfe et al. 1997) or as a chemical cue attracting higher trophic-level predators (Bonadonna et al. 2006, Dove 2015) warrants investigation into how DMSP production affects the ecological movement of CTX and other algal-derived toxins. Future studies will explore the relationship between the production of polyether biotoxins and DMSP within *Gambierdiscus* and other toxigenic dinoflagellate species and the role of DMSP in the trophic flux of microalgal toxins into marine food webs.

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The authors declare no conflicts of interest regarding this work.

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